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## Acute Toxicity and Immunotoxicity Testing of Total Petroleum Hydrocarbons in Aquatic and Terrestrial Organisms

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**Acute Toxicity and Immunotoxicity Testing of Total Petroleum  
Hydrocarbons in Aquatic and Terrestrial Organisms**

A thesis submitted in partial fulfillment of the requirements  
for the degree of Master of Science

By

**ANDREA R. HANDY**  
B.S., Wright State University, 1997

2007  
Wright State University

WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

May 30, 2007

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Andrea Renee Handy ENTITLED Acute Toxicity and Immunotoxicity Testing of Total Petroleum Hydrocarbons in Aquatic and Terrestrial Organisms BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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## Acknowledgements

I would like to thank the following for their contributions and efforts in this project:

J. Runkle Ph.D., K. Grasman Ph.D., G. A. Burton Ph.D, N. Bigley Ph.D., all of the students in Dr. Grasman's lab for their support and assistance, and Optech Operational Technologies Corporation and Wright Patterson Air Force Base U.S. Department of Defense STTR Program for funding and assistance.

I would also like to thank God, my family and friends for their encouragement and support during this course of study.

Sincerely,

Andrea

## Abbreviations

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BAF = Bioaccumulation Factor

BaP = Benzo[a]Pyrene

BSAF = Biota-to-Soil Accumulation

CD = Cell Differentiation Markers

CMI = Cell-Mediated Immunity

DCC = Differential Cell Count

DCF = 2',7' – Dichlorofluorescein Diacetate

FCA = Fluorescence Concentration Analyzer

FACS = Forward Angle Cell Scatter

H<sub>2</sub>DCFDA = Dichlorodihydrofluorescein Diacetate

HMI = Humoral-Mediated Immunity

II = Innate Immunity

LBSS = Lumbricus Balanced Salt Solution

LC<sub>50</sub> = Median Lethal Concentration

NBT = Nitro Blue Tetrazolium Dye Reduction

NMI = Non-Specific Immunity

NOEC = No Observed Effect Concentration

OC = Organic Carbon

PAH = Polycyclic Aromatic Hydrocarbons

PCB = Polychlorinated Biphenyls

RRBC = Rabbit Red Blood Cells

SRBC = Sheep Red Blood Cells

TCC = Total Cell Counts

TPH = Total Petroleum Hydrocarbons

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## **Thesis Outline**

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VI. General Methods

VII. Chapter 2

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## Abstract

The acute toxicity and immunotoxicity of JP-8 jet fuel on *Chironomus tentans*, *Hyallela azteca*, *Lactuca sativa*, *Eisenia foetida*, and *Lumbricus terrestris* was assessed using standard USEPA acute toxicity and static renewal toxicity tests. Three methods of spiking test soil with jet fuel were evaluated. In one method acetone was utilized as a carrier and the soil was dried in the fume hood; in another the soil was spiked directly with jet fuel and also was dried; and in the last the soil was spiked directly without drying. There was low survival in *C. tentans* in all treatments, including controls. There was significant mortality at 1500 ppm (AD soil) for *H. azteca*. Lettuce seed germination did not show any dose response. In contrast, there was a decreasing trend for lettuce root length in response to increasing JP-8 concentrations. We predicted lower mortality in worms exposed to soil treatments that were dried in the fume hood due to the loss of volatile toxic components. Nominal doses of jet fuel ranged from 0 ppm to 2000 ppm JP-8. Mortality was assessed on day 14. Although mortality varied among three experiments, soil that was spiked directly and not dried showed the highest levels of mortality when compared to soil treatments that were dried. Doses from 0 ppm to 750 ppm had low to moderate mortality (0-25%), while doses from 1000 ppm to 2000 ppm had high mortality (30-100%) for the no carrier/no drying soil treatment. Both soil treatments that were dried generally showed low mortality (0-15%), with the exception of the acetone carrier soil treatment in experiment 1 (which showed moderate to high mortality) 30% at 1500 ppm and 85% at 2000 ppm. To test immunotoxicity, coelomocyte counts were performed for controls and survivors of the acute toxicity test. There was a decreasing trend in immune endpoints (total cells, % viability, and total viable cells) as the jet fuel doses increased for the no carrier/no drying soil treatment when survivors of the 1000 ppm - 2000 ppm dose ranges were available for testing in experiments 1 and 2. Overall, high doses (1000-2000 ppm) of JP-8 produced mortality and immunotoxicity in redworms.

## **Chapter 1. Literature Review and Justification**

### **I. Introduction**

The main goal of this research was to develop a battery of assays that could be used to assess the acute toxicity and immunotoxicity of total petroleum hydrocarbons (TPHs) to various aquatic and terrestrial organisms. TPHs such as jet fuel and diesel fuel are used by the Air Force, airports, and gasoline companies. There is a potential environmental risk of spills when transporting and using these fuels. Previously contaminated sites and future spills may present opportunities for research on the effects of TPHs on aquatic and terrestrial organisms.

The Superfund Act requires that risk assessments be done at hazardous waste sites to determine the contamination of the sites, human health effects, ecological effects, and cleanup options. Toxic and hazardous substances can have a great impact on soil and water environments. Aquatic organisms such as *Chironomus tentans* and *Hyallela azteca* have been used extensively to assess the toxicity of various chemicals, including hydrocarbons. Terrestrial vascular plant tests are used for assessing the direct toxicity and contaminant uptake of chemicals (Pauwels, 1999). Lettuce seeds (*Lactuca sativa*) are commonly used for short-term soil toxicity tests because of availability, simplicity and low cost (Pauwels, 1999). Earthworms are also widely used test organisms for soil toxicity. Earthworms are common in soil and have a critical influence on soil structure, nutrient uptake, and plant growth (Edwards and Bohlen, 1996). Earthworms are vulnerable to impacts on soil, which makes them good bioindicators for chemicals in soil. Earthworms are also an important part of the food web and could contribute to bioaccumulation in higher trophic organisms (Grieg-Smith et al., 1992). Earthworms are easy to handle and cost-efficient test organisms.



Ecotoxicological assessments are used to characterize the biological effects of chemicals. Most involve acute toxicity testing, but many tests are being developed to assess sublethal toxicity such as behavioral, reproductive and immunotoxicity endpoints. The immune system is conserved phylogenetically, which allows earthworms to be used for immunotoxicity assays (Grieg-Smith et al., 1992). The function of the immune system is to protect the host against infectious agents. Many immunotoxicants suppress the immune system, which lowers host resistance, while others may stimulate certain responses and cause hypersensitivity (Lu, 1996). The relatively simple earthworm model has been studied extensively for decades. Many studies have demonstrated earthworm immune responses such as phagocytosis, agglutination, wound healing, cytolytic activity, and self-nonspecific recognition (Vetvicka and Sima, 1998). Consequently, these studies provide a sufficient basis for using earthworms to study the sublethal immunotoxic effects of chemicals.

Soil and water environments can be greatly affected by oil spills. The environmental relevance to oil spills and hazardous waste sites allows for interesting findings for eco-risk assessments. Total petroleum hydrocarbons contain various chemicals such as straight chain and branched alkanes and very volatile PAHs (aromatics). To evaluate the feasibility of using earthworm models to study immunotoxicity, the effects of PAHs and PCBs on earthworm immunity were reviewed. Jet fuel was used as the toxicant in this research project in order to add some valuable information to the literature. To justify using various immunoassays, studies that addressed various immunology endpoints were reviewed. Studies using different spiking methods were also reviewed to justify investigating various spiking methods used in this research project.

There are no definitive methods described in the literature for spiking soil with TPHs. Some constituents of TPHs are very volatile chemicals, and spiking with an acetone carrier requires allowing the acetone to evaporate for 24 hrs. This poses some questions about the actual concentration of chemicals in the soil because there is a potential for some of the chemicals to evaporate along with the carrier. However, when a carrier is not used, the small volumes of chemical added to large volumes of soil may not allow for uniform mixing. The actual concentrations of chemicals are extremely important when making evaluations of chemical exposure endpoints.

## **II. Literature Review**

### **Earthworm Models**

The major focus of this research project was on earthworms. Earthworms were among the first animals to have a closed circulatory system and a well-developed body cavity (Edwards and Bohlen 1996). By burrowing through the soil, earthworms mix it, form water-stable aggregates, increase soil macroporosity, aerate it, and improve its water-holding capacity (Edwards and Bohlen 1996). Earthworms are grown easily in the laboratory, making them a convenient test animal for evaluating the toxicity of pesticides and other chemicals in soil. They form an important part of the food web, serving as useful sentinel species for monitoring remediation. Some investigators have proposed that the immune functions of earthworms are sufficiently analogous to those in vertebrates for use in screening chemicals for immunotoxicity in higher animals (Edwards and Bohlen 1996). Earthworm model systems can be used to help understand the evolution of mechanisms of the immune system and possibly yield some information about the immunotoxicity of various chemicals to other organisms.

## Earthworm Toxicity Testing

Acute toxicity, reproductive, and behavioral testing with earthworms can be used to assess the effects of chemicals. Acute toxicity tests are short term tests that measure the effects of chemicals on survival of organisms (Hoffman et al., 1995). In typical acute toxicity testing protocols, earthworms are exposed to a chemical in artificial soil, and mortality is determined after 14 days. Mortality is expressed as a median lethal concentration ( $LC_{50}$ ), lowest observed effect concentration (LOEC), or no observed effect concentration (NOEC). Five dose concentrations and a control with 30 *Eisenia foetida* or 15 *Lumbricus terrestris* worms (3 replicates) per concentration are used in the test (Hoffman et al., 1995). For each concentration, the chemical is mixed with deionized water or acetone and spiked into the artificial soil (U.S. E.P.A., 1988). Spiking soil is a laboratory simulation of soil that has been contaminated by environmental spills.

Soils from contaminated sites can be used in acute toxicity testing for risk assessment. When hazardous waste soil samples are used, they are diluted to the appropriate concentrations by mixing with artificial soil (Office of Toxic Substances, 1988). These can be either fresh or weathered soils that can pose different hazards depending on the type of contaminants present and their bioavailability. Unfortunately, there has been little testing on organisms in weathered soils, so the potential risks are not known.

Another protocol for testing the acute toxicity of chemicals is the contact filter paper test. Standard filter paper is treated with chemicals of known concentrations. A carrier such as acetone is used to deliver the chemical to the filter paper. Ten earthworms are exposed individually to chemical residues for 48 hours to assess the relative toxicity of chemicals (Greig-Smith et al., 1992).

Acute toxicity testing categorizes chemicals, based on median lethal concentrations into 3 groups; “very toxic”, “intermediate toxicity”, or “non-toxic”.

An acute or chronic lab test must:

- (1) clearly define all components and be reproducible,
  - (2) ensure a close relation between the tested chemical and the earthworms, and
  - (3) eliminate interferences between this relation and the soil as a substrate
- (Hoffman et al., 1995).

Chronic tests must utilize an earthworm food. Presently, a type of synthetic food that will not interfere with the chemical tested is not available. Chemicals can bind to food in the intestinal walls which can alter the effects of the chemical to earthworms. Chronic tests expose organisms to a toxicant for one-tenth or more of their life cycle (Hoffman et al., 1995). They detect physiological disorders at sublethal doses corresponding to those in the field (Greig-Smith et al., 1992). When assessing reproductive abilities, earthworms are exposed to chemicals for 3 weeks. The number of cocoons produced is recorded, and they are incubated in untreated soil for 5 weeks to determine hatchability (Van Gestel et al., 1989). Acute and chronic toxicity tests durations can be quite long when trying to make ecological risk assessments.

Behavioral testing can range from 7 to 72 hours, which could help decrease the time necessary to assess the toxicity of chemicals. In behavioral testing, the avoidance response of earthworms to contaminated soil is tested in a circular fiberglass chamber. This test could help predict the responses of earthworms in acute and chronic toxicity tests (Stephenson, 1998).

### **Spiking Methods**

Various spiking methods are used to incorporate chemicals into soil, but there is little information on what method is the best for quality control. Several variables are involved when spiking soils such as soil moisture content, solvent or no solvent used, the volume of solvent, the volume of chemical, volume of soil, method of mixing, removal of solvent (partially, completely, or not at all), and the length of aging before the test is started. These variables are especially crucial when using volatile compounds and may cause differences between laboratory estimates and field conditions (Grant et al., 1994). Some of the methods have been used mainly for spiking single compounds. Solvents used for spiking soil include such as acetone, methanol, ethanol, and deionized water, depending on the compound that is being dissolved. Methods of mixing include hand mixing, household mixers, stainless steel rotating blenders, sieves, and reciprocal shakers (Kanaly et al. 1997, Meier et al. 1997, Baek 1993, Belfroid et al. 1993, Haque and Ebing 1988, and Van Gestel and Ma 1988). Unfortunately, there is little information on spiking with complex mixtures and volatile compounds. Gas chromatography is usually used to determine the amounts of chemical present in the soil. However, chemical analysis may not detect lower levels of chemicals that may still have a biological effect on organisms.

One study evaluated the biodegradation of a 5-ring polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP), by spiking a crude oil with [ $^{14}\text{C}$ ] BaP and unlabeled BaP. Six batches of soil were made where 20 g of crude oil containing BaP was added to

500 g (dry weight) of soil, mixed thoroughly and sieved through 5-mm openings. Homogeneity of the batches of soil was tested by combustion, which resulted in detection or recovery of 6.7% unlabeled BaP and only 0.0034% crude oil in the soil. The oil was composed of 15% compounds lighter than decane, which accounted for the volatilization that occurred during mixing (Kanaly et al., 1997). The results of this study raised questions about the effects of different spiking methods on the fate of a chemical in soil. The methods used to mix chemicals in soil can affect the actual concentration of chemicals in soil. Also, the types of chemical used such as volatile complex mixtures can affect the actual concentration of chemicals in soil.

### **Earthworm Immune System**

Earthworms were one of the first organisms to possess measurable immunological recognition and memory (Cooper et al., 1999). The immune cells (coelomocytes) of earthworms reside in the coelomic cavity, which runs the length of the worm and contains coelomic fluid. The coelomic fluid plays important roles in such as osmoregulation and immune defenses (Jarosz and Glinski, 1997). Functionally, coelomocytes resemble mammalian leukocytes, since they search out, phagocytize, and destroy foreign material. They also initiate inflammation, graft rejection, lysozyme production, and agglutinin secretion. Based on morphology, there are three major types of earthworm blood cells: hyaline amoebocytes, granular amoebocytes, and eleocytes. Hyaline amoebocytes include basophils and neutrophils; granular amoebocytes include granulocytes, type I and II acidophils, and transitional cells; and eleocytes are comprised of type I and II chloragogen cells (Jarosz and Glinski, 1997). Hyaline amoebocytes function in agglutination and graft rejection; granular amoebocytes function in inflammation and lysozyme production; and all cells function in phagocytosis except for chloragogen cells, which are believed to have some functions similar to the vertebrate

liver, but the exact immune function is not known (Valembois and Lassegues, 1995). Chloragogen cells contain granules (chloragosomes) that have enzymes and proteins. They are believed to contain heme enzymes such as catalase that may function as chloroperoxidase and may be involved in immune defense or xenobiotic transformation reactions. This could suggest that chloragogen cells also function as myelo-erythroid cells (Fischer, 1993). There are similarities and differences in earthworm immune cells and vertebrate immune cells. Although, earthworms lack immunoglobulins, they are believed to possess a non-self recognition system that may be an evolutionary adjunct to immunoglobulins (Cooper et al., 1999). In vertebrates, helper T lymphocytes (mostly CD3+, CD4+, and CD8-) and cytotoxic lymphocytes (mostly CD3+, CD4-, and CD8+) possess cell differentiation (CD) markers. Similarly, certain CD markers have been found in earthworms. Small electron dense cells (hyaline amoebocytes - stained by mouse antibodies) in earthworms were positive for the following CD markers: CD11a, CD45RA, CD45RO, CDw49b, and CD54 (Cooper et al., 1999). Other identified surface markers in earthworms are B2-microglobulin and Thy-1 (Cooper et al., 1999). In contrast, large electron dense cells were negative for all of these same markers. These similarities and differences help to indicate an evolutionary relationship in the immune systems of invertebrates and vertebrates.

### **Immunotoxicity Assays**

Toxic chemicals affect the viability, number, and functions of immune cells. Specific immune responses can be used as sensitive biomarkers for assessing the toxicity of chemicals. In general, high cell counts correlate with high cell viability and function. In acute and chronic toxicity testing total cell counts (TCC) and differential cell counts (DCC) are very effective ways to evaluate immune function in earthworms (Goven et al., 1993).

Immunoassays screen xenobiotics for suppression of humoral-mediated immunity (HMI), non-specific immunity (NSI), and cell-mediated immunity (CMI). These assays are relatively inexpensive and easy. HMI involves the elimination of extracellular pathogens by production of large numbers of antibody molecules specific for antigenic determinants on foreign pathogens. NSI involves the basic resistance to disease as a first line of defense until acquired immune response develops (phagocytosis). CMI involves specific recognition and selective elimination of foreign microorganisms and molecules (Kuby 1997). HMI can be assessed using hemagglutination of sheep red blood cells or secretory rosette formation with sheep or rabbit red blood cells. NSI response can be evaluated by testing phagocytosis of fluorescent beads or yeast cells and nitroblue tetrazolium (NBT) dye reduction. CMI function can be assessed with tissue transplantation and graft rejection tests (Venables et al., 1992). Three types of immunoassays may be employed to assess immunotoxicity. Tier I assays (toxicity and total cell counts) test initial exposure to chemicals and indicate chemical effects of acute toxicity (Venables et al., 1992). The chemicals that produced effects (e.g., mortality, low cell counts) in Tier I are tested at lower dose levels in corresponding Tier II assays, to assess sublethal effects such as low cell counts, cell viability, phagocytosis, and secretory rosetting (Luster et al., 1988). Tier III evaluates host resistance challenge by measuring levels and duration of pathogen replication, survivorship, and specific protective immune responses (Cooper et al., 1996). The tiered approach should allow efficient and cost-effective assessment of xenobiotic immunotoxicity and can also be valuable in public risk health assessment. Overall, these assays can provide valuable information about immunotoxicity in an efficient, cost-effective way.



## Studies in Phagocytosis

Phagocytosis is one of the primary nonspecific defense mechanisms in the animal kingdom. In earthworms, it is the basic mechanism for the clearance of intruders (bacteria, debris) in the body cavity fluid. Amoebocytes from many earthworm families participate in the various steps of phagocytosis, which involve non-specific recognition, chemotaxis, attachment, ingestion, and intercellular killing of biotic objects by lysosomal enzymes (Jarosz and Glinski, 1997).

Phagocytosis is a first line of defense in non-specific immunity, which makes it a good assay for endpoints in immunological studies. Phagocytosis in *L. terrestris* and *E. foetida* can be measured with flow cytometry, chemiluminescence, electron microscopy, and fluorometers. The coelomic fluid of *E. foetida* contains antibacterial substances such as lipoproteins that inhibit the growth of some bacteria. Upon infection, an increase in these antibacterial substances occurred in the coelomic fluid (Lassegues et al., 1989 as cited in Dales and Kalac, 1992). In the first few hours of exposure to pathogenic bacteria, secretion of antibacterial substances caused phagocytes to engulf bacteria (Dales and Kalac, 1992). The coelomic fluid of *E. foetida* has an opsonic role and significantly increases the rate of phagocytosis (Bilej et al., 1990). Opsonins are substances that coat bacteria and particles and render them more susceptible to phagocytosis (Cooper, 1976). Vaccination of *L. terrestris* or *E. foetida* with *Serratia marcescens* and *Aeromonas hydrophila* did not enhance the rate of phagocytosis compared to unvaccinated worms (Dales and Kalac, 1992). A phagocytosis rate of 2-4 bacteria per phagocyte per hour was found. This rate was low, but these experiments were conducted *in vitro* with medium that did not contain coelomic fluid (Dales and Kalac, 1992).

In *E. foetida* the phagocytosis rates of *Yersinia ruckeri* and *Aeromonas hydrophila* were not sufficient to prevent increase of either bacteria, which eventually increased to lethal concentrations. There was no significant difference in the increase of bacteria after 48 h for injection with smaller doses of *Serratia marcescens* (Dales and Kalac, 1992). From these results, it was concluded that species of pathogenic bacteria either reproduce well in coelomic fluid, produce exotoxins that reduce phagocytic capability, or are phagocytized at a lower rate than the reproduction rate. The primary line of defense against pathogens is phagocytosis, aided with antibacterial substances, and the opsonic role of coelomic fluid (Dales and Kalac, 1992).

### **Secretory Rosetting**

Secretory rosetting helps to evaluate whether or not cells have the ability to recognize non-self. The significance of rosette-forming cells in invertebrates is not as well known as in vertebrates. Rosette-forming cells in vertebrates possess receptors that recognize antigen and binds them to their surfaces (Cooper, 1976). In invertebrates, coelomocytes actively secrete a synthesized humoral agglutinin factor in response to antigens, which forms secretory rosettes (Eyambe et al., 1991). The percentage of rosettes formed with sheep red blood cells (SRBCs) vary with pH, temperature, and incubation temperatures.

The coelomocytes that form rosettes are nonadhering, nongranulated basophilic cells with a large nucleus and little cytoplasm. Vertebrates T-cells form spontaneous rosettes with SRBCs. Small electron dense coelomocytes resemble T-lymphocytes (various CD markers) in vertebrates and may have receptors for SRBCs (Toupin and Lamoureux, 1976). Thus, rosettes could possibly be evolutionary precursors of similar rosette forming cells in vertebrates.

## **Oxidative Burst Assays**

Several assays can be used to evaluate reactive oxygen species (ROS) in various cell lines. The NBT reduction assay can be used to evaluate the ability of earthworm coelomocytes to produce reactive oxygen species as an immune defense (Valembois and Lassegues, 1995). In vertebrates, phagocytosis stimulates ROS to exert a cytotoxic action on pathogens. ROS activity on lipids leads to an accumulation of lipofuscin brown bodies. Luminol-enhanced chemiluminescence (CL) was used to determine if earthworm coelomocytes possess ROS activity (Valembois 1995). NBT was also used to determine the cellular localization of ROS production and to confirm the CL results (Valembois 1995). Coelomocytes were separated into leukocytes and chloragocytes for the tests. Zymosan, EDTA, and bacteria were used to possibly stimulate an added response. Chloragocytes were more weakly stimulated by Zymosan than leukocytes. Catalase and sodium azide failed to inhibit the CL response of Zymosan stimulated leukocytes and chloragocytes. Zymosan stimulated leukocytes and chloragocytes also exhibited NBT reduction. Non-stimulated and stimulated chloragocytes were stained blue only when aggregated (not in isolation), and EDTA prevented them from aggregating so no NBT reduction occurred. Even though chloragocytes exhibited weaker responses than leukocytes in these tests, they both possess ROS activity (Valembois and Lassegues, 1995). Coelomocytes failed to exhibit ROS activity when phagocytizing synthetic HEMA particles and micrococci, which could indicate that phagocytized material was eliminated in another way (Bilej et al., 1991). This could also suggest that receptors are involved in recognition of particles, which may trigger ROS activity.

Fluorescent microplate assays can also be used to measure ROS activity. These assays allow several samples to be tested rapidly. The ROS activity of human polymorphonuclear leukocytes (PMNs) exposed to metals was evaluated in a microassay using the fluorescent probe dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (Molecular

Probes, Eugene, Oregon). ROS generation by PMNs was increased with exposure to metals (Ciapetti et al., 1998). H<sub>2</sub>DCFDA enters cells and is oxidized by ROS to fluorescent 2',7' -dichlorofluorescein-diacetate (DCF). The respiratory burst of phagocytes was evaluated using H<sub>2</sub>DCFDA and recorded using a fluorescence concentration analyzer (FCA) and forward angle cell scatter flow cytometry (FACS) (Rosenkranz et al., 1992). There was a linear relationship between oxidative products and cell counts. There was equal sensitivity in detection of oxidative products for both FCA and FACS (Rosenkranz et al., 1992). The two techniques differ in the type of oxidative products detected. It appears that FCA detects extracellular oxidative products, and FACS detects intracellular oxidative products (Rosenkranz et al., 1992). H<sub>2</sub>DCFDA was used with a human monocytic cell line (Mono Mac 6) and a murine macrophage cell line (J774) to evaluate ROS activity using a microplate fluorometer (Wan, Myung, and Lau 1993). Advantages to using the microplate assay include higher sensitivity (than NBT – nonfluorescent absorbance), multiple samples, rapid results, and the ease of using a microplate reader as opposed to a flow cytometer which requires more extensive training (Wan, Myung, and Lau 1993). Despite the advantages of microplate assays, there is currently no information in the literature using a fluorescent probe with the microplate in earthworms.

### **PAH Studies**

PAHs are planar molecules consisting of three or more benzene rings. They accumulate in the environment from spillage of oils and combustion of fossil fuels (Walker et al., 1996). PAHs are constituents of TPHs that accumulate in organic soils. Earthworms are prone to exposure to PAHs in contaminated soils (Van Brummelen et al., 1996). The PAH studies discussed here examined phenanthrene and fluoranthene in samples of contaminated forest soil from various sites near a blast furnace plant and soils spiked in the laboratory. PAHs are mutagenic and carcinogenic, and some are

immunosuppressive. Photodegradation, biodegradation, and volatilization remove PAHs. Bioaccumulation of PAHs in soil organisms (laboratory grown earthworms) and the role of soil fauna in PAH removal were examined (Ma et al., 1994). No earthworm mortality occurred with exposure to PAHs in soil. Earthworms enhanced the disappearance of PAHs in soil. The bioaccumulation of phenanthrene and fluoranthene was higher without food than with food, presumably reflecting the absorption of the chemicals to food in the digestive tract. Weathering of soil, which limits the bioavailability for uptake, may have reduced bioaccumulation of PAHs by earthworms (Ma et al., 1994). The second study focused on the distribution pattern of PAHs in soil and invertebrates (Van Brummelen et al., 1996). Profiles of fragmentation between soil layers, humus, and litter were evaluated to determine the levels of PAHs. BSAFs in fragmentation, humus, and litter were similar because PAH concentrations in the different soil layers were roughly the same. Many leaves were not decomposed enough to be palatable to earthworms, reducing feeding and decreasing the bioaccumulation of all 8 PAHs (including phenanthrene and fluoranthene) evaluated in the study. The PAH concentrations in earthworms were above background concentrations at sites closest to the blast furnace plant. This suggested a concentration-distance relationship for PAH contamination (Van Brummelen et al., 1996). In both studies, food intake decreased bioaccumulation of PAHs by earthworms. This could have been due to the digestion process in the gut. Differences in lipid content and recovery of PAHs could have been because soil contamination was done in the lab for Ma et al., (1994), and Van Brummelen et al., (1996) used field contaminated soil. The experimental protocols used in both studies were similar with a few minor exceptions.

## PCB Studies

PCBs are mixtures of congeners, and are found in manufacturing wastes, sewage sludge and dredged river sediments (Walker et al., 1996). The presence of chlorine in these molecules makes them very stable and lipophilic. PCBs have been evaluated in immunotoxicity studies with earthworms (Goven et al., 1993, Roch and Cooper, 1991, and Fitzpatrick et al., 1991). The reviewed PCB studies (Roch and Cooper, 1991 and Goven et al., 1993) both tested the effects of Aroclor 1254 on immunodefenses using the 5-day contact filter paper exposure method.

Exposure to Aroclor 1254 increased susceptibility to bacterial infection in *L. terrestris* but not *E. foetida*. In order to test earthworms susceptibility, *A. hydrophila*, were injected into *E. f. andrei*, and *L. terrestris*. In both Aroclor exposed and unexposed *E. f. andrei*, ten or one hundred bacteria produced 10-20% death. However, in those that received one million bacteria, 67% of exposed earthworms died, and 84% of unexposed earthworms died. In contrast, injection of one hundred bacteria in *L. terrestris* produced 37% death in exposed earthworms and no mortality in unexposed earthworms. The injection of one million bacteria in unexposed *L. terrestris* earthworms did not kill them, while 84% of exposed earthworms died after this injection. Thus, *A. hydrophila* was not pathogenic to unexposed *L. terrestris*, but was pathogenic to unexposed *E. f. andrei*. *L. terrestris* earthworms might have eliminated the bacteria by leukocyte-mediated reactions or antibacterial proteins. The sensitivity to the bacteria might have reflected a depression of macrophage function by Aroclor 1254. In *E. f. andrei*, the antibacterial proteins of chloragogue cells eliminated bacteria. Chloragogue cells are more abundant in *E. foetida* (cause yellow color of coelomic fluid). Aroclor 1254 inhibited leukocyte function (Roch and Cooper, 1991).

*Lumbricus terrestris* worms were exposed to 10 ug/cm<sup>2</sup> of Aroclor 1254, using the 5-day filter paper method (Goven et al., 1993). Aroclor 1254 increased the percentage of acidophils, and transitional cells and decreased the percentage of basophils, phagocytosis, erythrocyte formation, and secretory rosette formation. At 6 weeks, extruded cell counts (ECC) decreased in exposed earthworms. At 12 weeks, there were too few cells extruded to obtain accurate counts. Apparently, Aroclor 1254 decreased all immune parameters of coelomocytes collected prior to 18 weeks. At 18 weeks, when the concentration of Aroclor 1254 was lowest, ECC returned to control numbers, suggesting that depuration of the chemicals at 18 weeks allowed replenishment of the coelomic cavity (Goven et al., 1993).

PCB exposure causes coelomic leukocytes to secrete lower levels of agglutinins, and surface receptors for rabbit red blood cells in erythrocyte formation. This could account for the decreases in phagocytosis and wound healing (Roch and Cooper, 1991). Mammals exposed to PCBs have decreased immunoglobulin (Ig) Fc and complement receptors in phagocytic cells, and reduced Ig levels (Cooper 1976). Suppression of phagocytosis by coelomocytes could be homologous to suppression of mammalian cell phagocytosis. These studies suggest that earthworm immunotoxicity assays could be used to screen xenobiotics.

## Justification

Various studies that have used different soil spiking methods have not assessed their effects on acute and immunotoxicity to organisms. Spiking methods influence the concentration of chemicals in soil, which can alter the lethal and sublethal effects of on various organisms. Several different spiking methods are used to incorporate chemicals into soil (Kanaly et al. 1997, Meier et al. 1997, Baek 1993, Belfroid et al. 1993, Haque and Ebing 1988, and Van Gestel and Ma 1988). Most studies use single compounds. There is little of information in the literature about spiking complex mixtures and volatile chemicals.

A combination of tests using various organisms can give a comprehensive view of the effects of TPHs. The effects of petroleum spills can be assessed using aquatic and terrestrial organisms in acute toxicity and earthworm models in immunotoxicity testing with TPHs. Based on the reviewed studies and other studies, earthworms have proven to be sensitive bioindicator species for soil contamination, acute toxicity, and immunotoxicity testing. Mortality is a less sensitive endpoint than immunobiological coelomocyte-based endpoints (Viswanathan, 1994). Various assays in earthworm tests to measure immunotoxicity of chemicals have been done. However, there are no published studies on the immunotoxicity of total petroleum hydrocarbons on earthworms.

This study evaluated the effects of using three different spiking methods with JP-8 jet fuel. Chemical fractionation analysis was done on soil and sediment samples to determine actual concentrations of JP-8. This study assessed the effects of spiking method on acute toxicity testing in the following aquatic and terrestrial organisms: *Chironomus tentans*, *Hyallela azteca*, *Lactuca sativa*, *Eisenia foetida*, and *Lumbricus terrestris*. The immunotoxic effects of JP-8 on earthworms using various immunoassays such as secretory rosetting, oxidative burst, and phagocytosis were also assessed.



## Objectives

- a. To determine the differences in spiking methods using no carrier or acetone carrier with total petroleum hydrocarbons using standard USEPA acute toxicity and static renewal toxicity tests.
- b. To determine the acute toxicity of JP-8 jet fuel to aquatic and terrestrial organisms.
- c. To use a tier approach for assessing the sublethal immunotoxic effects of jet fuel on earthworms.

## Experimental Design

In order to attain the goals of this research project various methods and tools were used to assess the measurement endpoints. Three spiking methods were used to assess the effects of spiking methods on actual chemical concentrations in soil. Acute toxicity of JP-8 was assessed using USEPA Static Acute Toxicity Testing Protocols in various aquatic and terrestrial organisms. Also, various immunoassays were used to determine the immunotoxic effects of JP-8 on earthworms.

## General Methods

Three different spiking methods were used 1) direct without drying in the hood, 2) direct along with drying in the hood, and 3) acetone carrier with drying in the hood, to provide answers for the best method to use when spiking soils with volatile chemicals. Acute toxicity of JP-8 on *Chironomus tentans*, *Hyallela azteca*, *Lactuca sativa*, *Eisenia*

*foetida*, and *Lumbricus terrestris* was assessed using standard USEPA acute toxicity and static renewal toxicity tests. The acute toxicity tests were used for exposure to chemicals and to address how spiking method affects soil toxicity, and acute toxicity. Further specific aims were used to assess the immunotoxicity of chemicals on the survivors of these tests and address how sublethal doses of JP-8 jet fuel suppress immune responses in earthworms. In order to evaluate the effects of JP-8 jet fuel on the earthworm immune system, a battery of immunotoxicity assays was used. The various immunotoxicity assays were phagocytosis using the Molecular Probes FluoroReporter™ assay kit, oxidative burst, and secretory rosetting. These assays helped to evaluate the immune suppression caused by exposure to JP-8 jet fuel.

## Chapter 2.

### Evaluation of Three Spiking Methods in Acute Toxicity Testing of JP-8 Jet Fuel in Aquatic and Terrestrial Organisms

#### Summary

This experiment determined whether different spiking methods affect amounts of JP-8 delivered to soil and thus toxicity to various organisms. Standard USEPA protocols were used for acute toxicity testing of *Chironomus tentans*, *Hyallela azteca*, *Lactuca sativa*, *Eisenia foetida*, and *Lumbricus terrestris*. Immunotoxicity also was assessed in earthworms. One spiking method utilized acetone as a carrier and was dried in the fume hood for 24 hours (acetone dried - AD); another spiked the soil directly and also was dried (non-acetone dried - NAD); and the third spiked the soil directly without drying (no acetone, not dried - NAND). Chemical fractionation analysis detected no total TPHs ( $\geq C8$  -  $\leq C35$ ) in the pre-testing sample for AD soil at the highest nominal dosage of 1500 ppm. The actual concentration of TPHs for NAD soil was 79% lower than the nominal 1500 ppm dosage for the pre-test sample. The pre-test sample for NAND soil had an actual concentration of TPHs that was 52% lower than the nominal 1500 ppm dose. Overall, NAND soil retained the highest actual concentrations of TPHs. There was low survival in *C. tentans* in all treatments, including controls. There was significant mortality at 1500 ppm (AD soil) for *H. azteca*. Lettuce seed germination did not show any dose response. In contrast, there was a decreasing trend for lettuce root length in

response to increasing JP-8 concentrations. *E. foetida* had higher mortality than *L. terrestris* for NAND soil. As the JP-8 concentration increased, mortality in *E. foetida* increased in NAND soil. The lowest earthworm mortality occurred in soils that were dried due to loss of volatile toxic components. Overall, acetone dried soils had lower actual concentrations and lower mortality than non-acetone dried soils.

## **Introduction**

Total petroleum hydrocarbons (TPHs) such as jet fuel and diesel fuel are used by the military, airports, and gasoline companies. Transportation spills, urban runoff, and industrial and municipal discharges account for 35% of the total oil discharged onto land and freshwater environments (Albers, 1995). For example the Exxon Valdez Spill oiled about 16% of the Prince William Sound shoreline (Boehm et al., 1996).

Acute and chronic effects of petroleum hydrocarbons on invertebrate and plant species include death, reduced growth and development, immunotoxicity, local population changes, and altered community structure (Albers, 1995). In acute toxicity testing, aquatic and terrestrial organisms are exposed to a chemical in sediment or soil from 5-14 days according to standard protocols. Sensitivities to TPHs vary among aquatic and terrestrial organisms. The direct and photoinduced sediment toxicity of four (acenaphthene, phenanthrene, fluoranthene, and pyrene) polycyclic aromatic hydrocarbons (PAHs) was studied in the marine amphipod *Rhepoxynius abronius* (Swartz et al., 1997). The toxicity of fluoranthene and pyrene to survivors of direct toxicity tests was increased

with UV radiation exposure, but the toxicity of acenaphthene and phenanthrene was not affected due to the phototoxic molecular descriptor range (Swartz et al., 1997).

Spiking soil in the laboratory is used to simulate contamination by environmental spills. There are no definitive soil spiking methods for volatile compounds to mimic the effects of TPH spills in the laboratory. Sediments are commonly spiked wet with a carrier solvent such as methanol or acetone (Lotufo and Fleeger 1996, Landrum et al., 1997). Various spiking methods with and without solvents are used to incorporate chemicals into soil, but there is little information on what method is the best for incorporating the highest level of chemical into soil. Several variables affect delivery of chemicals to soils when spiking, including use, choice, and volume of solvent, volume of chemical, volume of soil, method of mixing, removal of solvent (partially, completely, or not at all), length of drying before the test is started, and soil moisture content (Grant et al., 1994). These variables are especially crucial when using volatile compounds and may cause differences between laboratory estimates and field conditions (Grant et al., 1994). Common solvents for spiking include acetone, methanol, ethanol, and deionized water. Methods of mixing range from hand mixing, household mixers, stainless steel rotating blenders, sieves, and reciprocal shakers (Haque and Ebing, 1988, Van Gestel and Ma, 1988, Belfroid et al., 1993, Baek, 1993, Kanaly et al., 1997, and Meier et al., 1997).

Spiking small volumes of chemicals into large volumes of soil and some methods of mixing may cause uneven distribution of the chemical in the soil. This could cause inconsistent exposure and biological effects in toxicity testing. Solvents or carriers are

often used to distribute evenly small volumes of chemicals into soil. Carriers must be evaporated, usually in a fume hood, and the soil must be re-hydrated. Evaporation of the carrier in the fume hood can also allow the volatile constituents to evaporate, causing uncertainty about the actual concentration of chemical in the soil. Unfortunately, there is little information on spiking with complex mixtures and volatile compounds.

Several quick (5, 7, and 14 days) and inexpensive bioassays can be used in laboratories to assess the biological effects of contaminants on freshwater aquatic species such as *Hyaella azteca*, and *Chironomus tentans*, and terrestrial species such as *Lumbricus terrestris*, *Eisenia foetida*, and *Lactuca sativa* (lettuce seeds). Soil toxicity testing is a relatively new field when compared to aquatic toxicity, which allows for a greater choice of species and life stages when testing toxicity of chemicals (Pauwels, 1999).

Terrestrial soil toxicity allows the assessment of soil contaminant bioavailability. Earthworms are exposed to soil contaminants dermally and through ingestion of soil, making them good bioindicators of the effects of hazardous chemicals on the terrestrial environment (Edwards and Bohlen, 1996). Although, there are very significant differences between vertebrates and earthworms, the phylogeny of the immune system can account for similar functions. The coelomocytes of earthworms eliminate foreign materials efficiently and possess defense mechanisms such as graft rejection (similar to vertebrate leukocytes), which makes them good indicators for studying the immunotoxic effects of hazardous chemicals (Hostetter and Cooper, 1974).

Few studies have examined the effects of different spiking methods on the toxicity of total petroleum hydrocarbons on aquatic and terrestrial environments. The objective of this study was to determine the effects of using different spiking methods on the amounts of JP-8 jet fuel incorporated into soil and thus toxicity to aquatic and terrestrial organisms. Three different spiking methods were evaluated. One spiking method utilized acetone as a carrier and was dried in the fume hood for 24 hours (AD); another spiked the soil directly and also was dried (NAD); and the third spiked the soil directly without drying (NAND). Both aquatic and terrestrial organisms were used to testing with multiple species and test media (soil vs. sediment).

## **Materials and Methods**

### **Soil Spiking**

Soil from the banks of the Mad River (Greene County, Ohio) was used as the testing medium (soil or sediment) for acute toxicity testing with aquatic and terrestrial organisms. The Mad River habitat consists of locust trees, fox grapes and black willows (Combs and Gillen, 1994). Organisms were exposed to seven nominal doses (0, 10, 100, 250, 500, 1000, 1500) of JP-8 in either sediment or soil. Controls were handled identically to spiked soils. All control soils (AD, NAD, and NAND) were shaken for 24 hours. Additional Trout Farm sediment was used as a control for aquatic toxicity testing. The AD control received acetone and was dried in the fume hood for 24 hours. The NAD control did not receive acetone but was dried in the fume hood for 24 hours. The NAND control did not receive acetone and was not dried. The soil was spiked with JP-8 jet fuel

using three methods. Acetone was used as a carrier in AD soil, which required drying in the fume hood and re-hydration. NAD soil was spiked directly (without acetone carrier) and dried in the fume hood. NAND soil was spiked directly without drying in the fume hood. Acetone was added as a carrier for each dose based on a 10% volume soil ratio of acetone for the highest dose of JP-8. The appropriate amounts of soil were weighed and placed in 3.785 liter (1 gallon) plastic containers. Soil for each concentration was spiked all at once. The soil was spiked with the appropriate amount of JP-8 jet fuel, the containers sealed and agitated manually. The containers were shaken mechanically at 300 rpm for 24 hours. AD and NAND soils were spread out onto aluminum foil and dried for 24 hours in the fume hood. After drying, the soil was broken into small clumps with a rolling pin and re-hydrated with double distilled water. The pH and moisture of each treatment were measured using a Kelway soil acidity and moisture meter.

### **Culturing and Collection of Organisms**

*Chironomus tentans* and *Hyalella azteca* were shipped via overnight express to the Institute for Environmental Quality at Wright State University, Dayton, Ohio, from Aquatic Research Organisms (Hampton, Massachusetts, USA). Upon arrival, organisms were placed in aerating culture dishes and examined for overall health. Only the most robust individuals were handpicked for testing. Lettuce seeds were also randomly picked for testing. Mature clitellate earthworms were purchased from Carolina Biological Supply and acclimated in the dark to  $20 \pm 2^{\circ}\text{C}$  (*E. foetida*) or  $10 \pm 1^{\circ}\text{C}$  (*L. terrestris*) in an environmental chamber for at least 2 weeks before testing.



### Static Renewal Sediment Toxicity Bioassay

The amphipod, *Hyalella azteca* (7-14 days old), and the midge, *Chironomus tentans* (8-12 days old), were exposed to 11 sediment treatments (*i.e.*, 4 control treatments of sand, trout, AD, and NAD soil, and six treatments spiked with JP-8) during a 7 day static renewal sediment bioassay (USEPA, 600/4-91/002, 1994). Two water renewals were performed per day with a Zumwalt diluter system (*i.e.*, AM and PM water renewal). All treatments contained 85 g (100 ml) of sediment. Both Mad River soil and Trout Farm sediment were used for bioassays. Trout Farm sediment was used as an additional control for aquatic toxicity testing to compare sediment suitability in different media. Each sediment treatment was placed into a 250 ml beaker (replicates of 3 for each concentration). One hundred and fifty ml of laboratory culture water (hardness ranging from 160-180) was added to each beaker and allowed to settle for 24 hours. On day 0 and 7, overlying water was removed from treatment beakers for initial and final chemical analysis (e.g., pH, hardness, total ammonia, alkalinity, dissolved oxygen and conductivity). On all other days, temperature and dissolved oxygen were measured daily. On day 0, once water chemistry parameters were measured, particulates had sufficiently settled, and the morning water change was completed, all test organisms (10 of each *C. tentans* and *H. azteca*, in the same beaker) were gently added to the test water in each beaker. Air lines were applied to each system to ensure dissolved oxygen levels would not reach below 4.0 ppm as standardized by US EPA protocol. Water temperature was kept at  $23 \pm 1^{\circ}\text{C}$ . Following the morning water change each day, 1.5 ml of Tetrafin food slurry was supplied to each beaker according to US EPA guidelines. At day 7 of

exposure, all replicate beakers were monitored for organism survival and water quality. The contents of each test beaker were sieved with a No. 45 ASTM sieve to collect and then enumerate surviving organisms.

### **Lettuce Seed Germination Success and Root Elongation Bioassay**

Lettuce seeds were exposed to 10 treatments (*i.e.*, 3 control treatments (AD, NAND, and Artificial Soil) and six treatments spiked with JP-8) during a 5 day soil bioassay (USEPA, 1988). Each treatment consisted of 40 g Mad River Soil placed into a 100 mm x 15 mm plastic petri dish. In each dish the soil was moistened with 8-10 ml of Milli-Q water, and ten lettuce seeds were uniformly distributed less than an eighth of an inch below the soil (each treatment was done in triplicate). No additional water was added throughout the test, as the petri dishes were kept sealed. The lettuce seeds were subjected to continuous fluorescent light for 5 days. After the 5 day exposure, all dishes were examined for lettuce seed germination success (%) and seed root elongation (mm).

### **Microtox<sup>®</sup> Solid-Phase Test**

The Microtox<sup>®</sup> Solid-Phase Test was performed on 6 treatments (*i.e.*, 2 control (AD and NAND) treatments and 4 soils spiked with JP-8) with a Microtox Model 500 (Microbics Corp., 1992). The detailed test protocol provided by Microbics was followed.

### **Earthworm Soil Toxicity Testing**

Acute toxicity tests were done in triplicate with either 10 *E. foetida* (redworms) (200-300mg) or 5 *L. terrestris* (nightcrawlers) ( $\geq 3$ g) and 200 g dry weight of soil per 1 pint mason jar (USEPA, 1988). For all soil tests, soil moisture and pH (target range 6-8)

were measured at the beginning and end of the test. Both species were exposed to 8 treatments with an acetone carrier and drying (2 controls - AD and NAND and 6 concentrations of JP-8), and 7 treatments (1 control and 6 doses of JP-8) for direct spiking with drying and direct spiking without drying. Earthworms were randomly selected and placed into each treatment jar. Temperature was maintained at  $20 \pm 2^{\circ}\text{C}$  for *E. foetida* and  $10 \pm 1^{\circ}\text{C}$  for *L. terrestris* in environmental chambers. Continuous light was used to encourage burrowing of worms. Mortality was assessed after 7 and 14 days of exposure.

### **Immunotoxicity Testing**

Both *E. foetida* and *L. terrestris* were rinsed with  $4^{\circ}\text{C}$  saline to remove soil. *E. foetida* cells were obtained using the extrusion method. Two *E. foetida* were placed into a microcentrifuge tube with 0.5 ml of extrusion fluid for 4 minutes. Extruded cells in the extrusion fluid were diluted with 0.5 ml of *Lumbricus* Balanced Salt Solution without  $\text{CaCl}_2$  (LBSS w/o  $\text{CaCl}_2$ ) (Brousseau et al., 1994).

The puncture method was used to obtain cells from the *L. terrestris*, a sharpened Pasteur pipette was inserted laterally into the area posterior (approximately 5-10 segments) to the clitellum. Coelomic fluid flowed into the pipette by intracoelomic pressure. Cells were diluted with 0.5 ml of *Lumbricus* Balanced Salt Solution without  $\text{CaCl}_2$  (LBSS w/o  $\text{CaCl}_2$ ) (Brousseau et al., 1994). Cells for *E. foetida* and *L. terrestris* were recovered by centrifugation at  $150 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  and resuspended in 0.5 ml of LBSS w/ $\text{CaCl}_2$  at  $4^{\circ}\text{C}$ . For total cell counts, fifty  $\mu\text{l}$  of cells were transferred into a test tube containing 450  $\mu\text{l}$  of LBSS w/ $\text{CaCl}_2$ , and 500  $\mu\text{l}$  of trypan blue viability dye. The cells

were allowed to absorb the dye for 10 minutes at room temperature. An aliquot of 25  $\mu$ l of cells was placed into the hemacytometer, and the cells were counted at 400x. The following endpoints were assessed: number of total coelomocytes, number of live coelomocytes, and percent viability of coelomocytes.

A drop of the diluted cells was placed on a slide (using a disposable pipette) in order to make a smear. After the slide dried, it was stained with Wright stain (Sigma, St. Louis, Missouri) and cell types were counted at 1000x using oil immersion.

### **Chemical Analysis**

The National Total Petroleum Hydrocarbon Working Group has recommended that the optimal approach for evaluating total petroleum hydrocarbons is by breaking the product into seventeen representative size fractions based on carbon length, and identifying which are aliphatic or aromatic (Lancaster, Pennsylvania, USA). It appears that the environmental fate and effects is strongly linked to the size of the compounds. Since petroleum products contain a multitude of chemical compounds and vary widely in their composition, this size fractionation approach provides a more systematic and accurate approach for environmental assessments. Seventeen representative soil and sediment samples were selected for TPH analysis with Lancaster Laboratories (Table 2.1) (Lancaster, Pennsylvania, USA). Twelve samples were sent as pre-test (before they were used for bioassays) and five were sent as post-test (sample remaining after bioassays). Thirteen of the samples collected for analysis were soils, and four were sediments. Soil samples were collected directly following spiking procedures (See Soil Spiking above). Sediment treatments were comprised of spiked soils, but were placed in beakers with 150

ml of overlying test water. The pre sediment samples were underwater for a day before being sent for analysis and the post sediment samples were collected on the final day of the sediment bioassay.

## **Statistical Analysis**

The combined effects of different spiking methods were analyzed using two-way analysis of variance (ANOVA) with interaction (Statistica, Statsoft, Tulsa, OK). The non-parametric Jonckheere test for ordered alternatives was used to evaluate monotonic trends between dose groups and dependent variables (Hollander and Wolfe, 1973). Dunn's test was used to compare each dose to controls when the Jonckheere test indicated a significant dose response. The Dunn's test indicated one-way differences between control groups and other groups. Both the Jonckheere and Dunn's test allowed for unequal sample sizes and are corrected for ties.

## **RESULTS**

### **Chemical Analysis of Spiked Soils**

Although only TPH pentane extract levels are discussed, results from all four ranges are presented (Table 2.1).

In the following results and discussion, spiked treatments are referred to by the nominal, or target, concentrations of JP-8. It is apparent that these nominal values grossly overestimated actual measured concentrations (Table 2.1). Chemical analysis was conducted for all doses (pretest samples) of the acetone-dried soil to evaluate the loss of

JP-8 with drying in the fume hood. Seventy-one ppm TPH ( $\geq C_{21}$  -  $\leq C_{35}$ ) was detected from the 0 ppm AD treatment sample. No other control treatment samples yielded measurable TPH concentrations. For the AD soil, both the 10 and 100 ppm soil samples had no detectable TPH levels. Although the actual concentrations were low, detected total TPH actual concentrations increased from 250 to 1000 ppm AD treatments. The 1000 ppm AD pre-test soil sample had a total TPH concentration of 160 ppm, which was approximately 86% lower than the nominal concentration of 1000 ppm. Both the acetone dried 1500 ppm soil pre- and post-samples showed no detectable TPH levels (Table 2.1).

The air drying process caused significant loss of TPH levels within a 24 hr period (particularly of carbon sizes  $\leq C_{12}$ ). The NAD pre-test 1500 ppm soil sample had lower actual concentrations (38-100% in fractions ranging from C8-C16) than NAND pre-test 1500 ppm soil samples (Table 2.1). TPH detections at the end of the NAND bioassay exposures several days (5-14) later showed losses (based on pretest levels) ranging from 75 to 100% (non-detectable levels), with the degree of detection being lower in the smaller-sized fractions (Table 2.1).

The only sediments chemically analyzed were the pre- and post- 1500 ppm AD and NAND samples. Low levels of the smaller, more volatile fractions were detected (7 to 240 ppm) and decreased to 0 to 9 ppm in post- (*i.e.*, by test termination) treatments (Table 2.1). However, there was a large loss of TPH s from the pre to post 1500 ppm NAND soil sample, relative to the same NAND sample of sediment. Both the pre- and post- 1500 ppm AD samples yielded no TPH, but decreasing levels of TPH from pre to

post samples were detected for the 1500 ppm NAND soil samples (Table 2.1). The percentage loss for sediment treatment samples was less than soil treatment samples. However, overall levels of TPH for these sediments samples were lower than soils at the test initiation. This could be due to sediments being covered by water, which could have resulted in less evaporation of JP-8.

### **Sediment Toxicity Test - *Chironomus tentans***

The *C. tentans* appeared in good health before testing. However, survival was relatively low for most spiking methods (Figure 2.1). Despite the marginal survival and growth of the controls and lower JP-8 concentrations, there was a significant dose effect (ANOVA) in the 1,500 ppm NAND treatment with 0% survival. No significant growth effects were observed at lower nominal TPH concentrations. It was observed that those *C. tentans* surviving the 7 day exposure were unable to create a characteristic chironomid “case” in which to live throughout the test period. *C. tentans* survival and growth were highest in the Trout Farm sediment (70%). High survival in the Trout Farm sediment, but not in Mad River Soil controls (NAD soil 50% and AD soil 30% survival), could have been another good indication of artifacts resulting from sediment suitability. Although Trout Farm sediment had higher survival than the other controls there was no significant difference when controls were analyzed separately (Jonckheere), so all controls were pooled for exposure response (Jonckheere).

### **Sediment Toxicity Test - *Hyallela azteca***

All *H. azteca* controls had similar high survival and were pooled for exposure - response analysis. The *H. azteca* appeared extremely healthy upon arrival and displayed high survival for most of the treatments, including controls (Fig. 2.2). There was a slight decrease in survival at 500 ppm AD and higher, but survival differences were not significant (ANOVA) until the 1500 ppm NAND treatment. Survival was never lower than 63.3% for any JP-8 treatment. However, there was relatively low (63.3%) *H. azteca* survival following exposure to control AD (Mad River Soil).

Most water chemistry measurements for the 7 day exposure period were within acceptable ranges. Initial and final total ammonia levels for the Trout Farm Control treatment were relatively high (initial was 14.7 mg/L and final was 15.2 mg/L). However, both *C. tentans* and *H. azteca* displayed high survival in the Trout Farm Control. Mean dissolved oxygen was greater than or equal to 6.88 mg/L and mean temperature was  $23 \pm 1^{\circ}\text{C}$  for all treatments.

### **Lettuce Seed Germination Success and Root Elongation**

Mean lettuce seed germination did not show any dose response with increasing JP-8 concentration (Fig. 2.3). Percent germination was 86.7% or higher for all treatments (93.3 % or higher for all control treatments) with no effect of JP-8.

The root growth measurements, however, clearly displayed a dose response with increasing JP-8 concentration (NAND and AD soils). The artificial soil yielded the



highest mean root length (55.0 mm), perhaps indicating again that the Mad River soil is not the best quality soil even without contaminants. Both 1500 ppm AD and NAND treatments yielded the lowest mean root length equating to reduced growth levels of 55 and 52.4% when compared to artificial soil controls (ANOVA).

### **Microtox<sup>®</sup> Solid-Phase Test**

There were no clear indications of toxicity for this test. When compared to the Mad River Soil Control NAND, all other NAND treatments show lower EC<sub>50</sub> concentrations. However, when compared to the AD soil control, all other treatments except the 1500 ppm AD have higher EC<sub>50</sub> concentrations. Subsequently, this also means that the Mad River Soil Control AD treatment was more toxic than the control Mad River soil treatment without acetone.

### **Earthworm Bioassays**

#### *Acute Toxicity Test Results – 14 d Mortality*

Mortality was relatively low (0-5%) in the control treatments (Table 2.2). There was a significant difference ( $p < 0.001$ ) for mortality in both species of worms for dose and carrier interaction effects. The no carrier without drying treatment had the highest level of mortality in groups exposed to JP-8, with the no carrier with drying and acetone carrier and dried treatments having very similar (lower) mortality results.

For *L. terrestris*, the NAND spiked soils yielded 0% mortality for 0 – 500 ppm, 26.7% mortality at 1000 ppm and 100% mortality at 1500 ppm JP-8 (Fig. 2.5). Actual concentrations suggested a *L. terrestris* TPH dose effect level (LD<sub>50</sub>) of approximately 200 ppm (extrapolated from graphs and chemical analysis). The mortality responses of *E. foetida* were higher than *L. terrestris*. *E. foetida* exposed to NAND soil had 10% mortality in controls and 100 ppm, 20% mortality in 10 ppm, 30% mortality in 250 ppm, 70% mortality in 500 ppm, and 100% mortality in 1000 and 1500 ppm JP-8 (Fig. 2.4). Actual concentrations suggested an *E. foetida* TPH dose effect level (LD<sub>50</sub>) of approximately 70 ppm (extrapolated from graphs and chemical analysis). *L. terrestris* and *E. foetida* exposed to soil directly spiked with drying, and acetone dried soil had no significant mortality at any dose.

## **Immunotoxicity in Earthworms**

Interaction and dose effects were not significant ( $p > 0.05$ ) for cell counts and viability in *E. foetida*. There was a significant difference ( $p < 0.05$ ) for spiking methods (carrier effect) when comparing total cells, percent viability, and total viable cells for *E. foetida*. The NAND and NAD soil treatments yielded very similar results. The values for the NAND and NAD soil treatments were 15-20% higher than the AD soil treatment values based on 2-way ANOVA results. Results from Jonckheere indicated a significant ascending trend in immunotoxicity endpoints for *E. foetida* exposed to AD soil. This suggested that the AD soil treatment caused not only low mortality, but also lower immunotoxicity endpoints.

In contrast, there was not a significant difference ( $p>0.05$ ) when comparing these same immunotoxicity endpoints for interaction, dose and carrier effects in *L. terrestris*. *L. terrestris* had the lowest cell counts for the NAD control soil. The immunotoxicity endpoints were higher than the controls at the 100 and 250 ppm doses and lowest at the 500 ppm, and 1000 ppm for all treatments. Overall, there was no evidence for immunotoxic effects in *L. terrestris*, which was probably due to low mortality (Table 2.2).

## DISCUSSION

There were significant differences in TPH concentrations between the three spiking treatments. The degree of TPH loss varied both by treatment and by carbon fraction size. Direct spiking without drying yielded the highest actual concentrations of JP-8. Crude oils exposed to air, water, or sunlight evaporate readily (Hoffman et al., 1995). The evaporation rate for heavy crude and refined oil products has been reported to be approximately 10%, whereas the level of evaporation can be as much as 75% for light crude and refined oil products in the atmosphere (air, water, or sunlight can alter the composition) (Hoffman et al., 1995). The total TPH detection for the control AD pre-test soil sample could have resulted from other extractable organics (not JP-8), since the detected fraction ( $>C_{21} - \leq C_{35}$ ) was not present in JP-8. Direct spiking appears to be the best spiking method for retention of JP-8 in soil, as well as acute toxicity. Spiking with small volumes for low concentrations did not pose problems. The complexity of soil and sediment systems which have several binding sites can make it difficult to distribute chemicals evenly (Northcott and Jones, 2000). The amount of chemical retained in soil or

sediment affects the biological impacts to organisms. Chemical analysis may underestimate the biological effects of chemicals. In general, results for mortality were consistent for the various organisms.

The overall low survival of *C. tentans* in the 7-day exposures could have been due to a number of conditions independent of JP-8 exposure. Overall organisms' health, contrary to outward appearances, might have been poor following shipment. Test organisms that are shipped from a biological supply company have a greater chance of encountering stress prior to the test exposure than do those organisms coming directly from a culture in the facility where the test is being conducted. In addition, the sand control and the Mad River Soil used for all spiked treatments and two control treatments could have been unsuitable substrates for *C. tentans* survival even without contaminants (*i.e.*, spiked with JP-8).

*Hyallela azteca* showed significant effects at the 1500 ppm (NAND) dose and minimal effects at other doses. High *H. azteca* survival overall might indicate that large amounts of JP-8 volatilized before significant exposure could take place at lower nominal doses. Effects at higher nominal doses make *H. azteca* a more suitable indicator of TPH toxicity than *C. tentans*.

The lettuce seeds appeared to be in excellent condition prior to testing, and most seeds germinated within a day of the beginning of exposure. This measurement endpoint tends to be less sensitive to chemical contaminants because the seed covering and internal

growth process could minimize the influence of external contaminant exposure. This could account for the results of no clear dose effect for seed germination. Based on the effects at the nominal 1500 ppm dose for AD and NAND soils, root growth measurement appears to be a more sensitive endpoint. There were similarities in mean root length for the 1500 ppm AD and NAND soils. This could have been due to changes in soil characteristics for the AD soil or more volatilization in lower concentrations of AD soil.

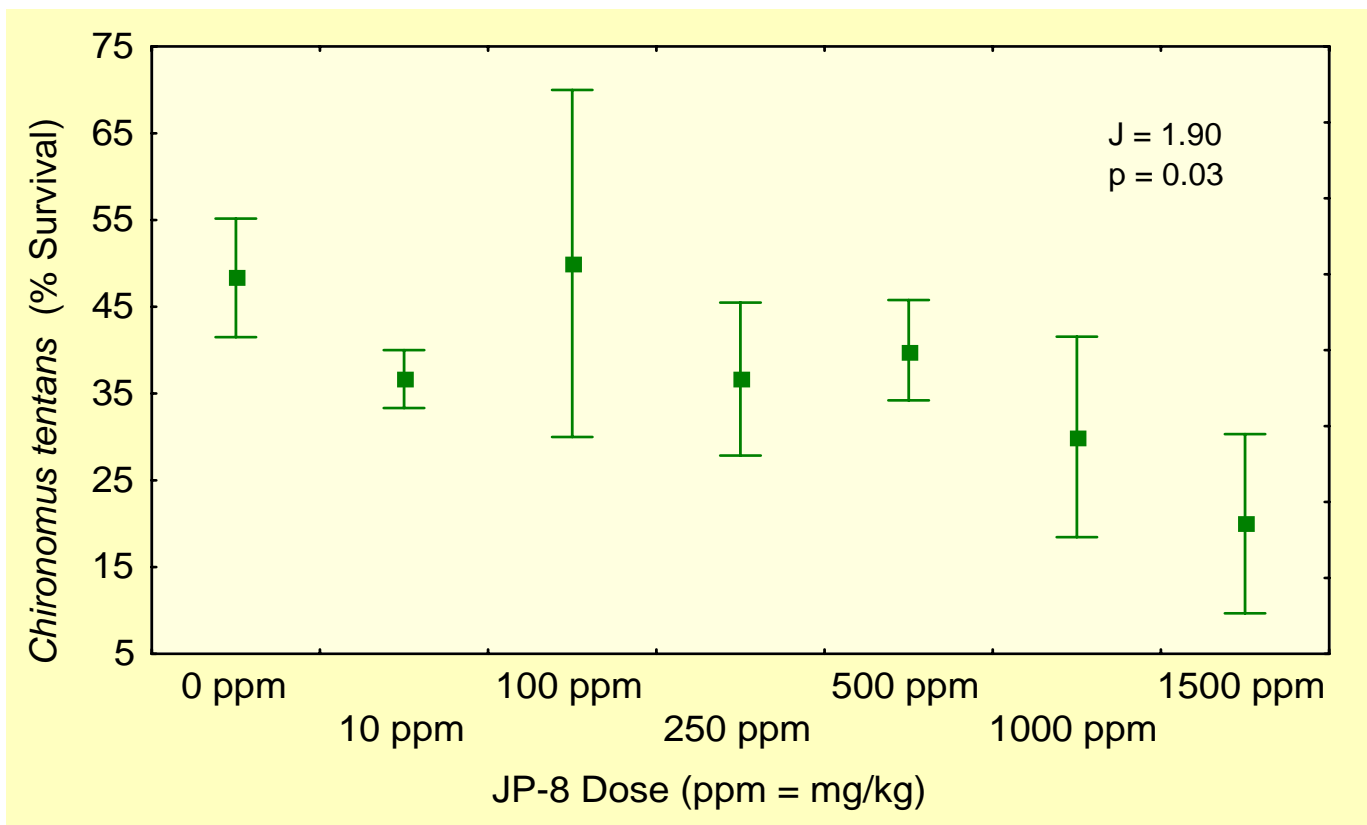
Overall, *E. foetida* had higher mortality than *L. terrestris*. For *E. foetida* there was a suggested dose effect at approximately a four-fold lower concentration (~34 -50 ppm) of TPHs than *L. terrestris*. Species sensitivity could account for differences in mortality results between both species. *L. terrestris* are larger than *E. foetida*, which could result in a lower surface area to volume ratio (body burden). Overall, *E. foetida* appear to be a more sensitive indicator of TPH toxicity than *L. terrestris*. Nominal mortality for both species of worms occurred in AD and NAD soil, even in the 1000 and 1500 ppm nominal doses. This may have resulted from increased volatilization due to drying in the fume hood. The increasing mortality with increasing nominal dose of *E. foetida* exposed to NAND soil was due to the higher retention of JP-8 (less volatilization with no drying). In the 1500 ppm NAND treatments, there was 100% mortality for both worm species. Survival was greater in other treatments, which was probably due to volatilization of JP-8 in soils that were dried.

The immunotoxicity results did not support our hypothesis of differences between doses and spiking method. In the other experiments in our laboratory using NAND soil, dose effects began at 500 ppm, and there were survivors at the 1000 ppm and 1500 ppm doses, which resulted in a decreasing trend of immunotoxic effects at 1000 ppm and above. In the present experiment, high mortality at and above 1000 ppm precluded measurements of immunotoxicity endpoints in both worm species. There was no indication of a trend for *L. terrestris* immunotoxicity endpoints at lower doses. Lastly, there was not a totally clear dose response for all endpoints in *E. foetida*. Drying soil in the fume hood and acetone decreased the toxicity of JP-8 in AD soil treatments. Low retention of JP-8 in AD and NAD soils also decreased immunotoxic effects of JP-8. These alterations in AD soil may have contributed to the ascending trend exposure response for immunotoxicity endpoints in *E. foetida*. Also, immune stimulation may have occurred in *E. foetida* exposed to AD soil. For both species of worms, there was a slight decrease in total cell counts, total viable cells, and % viability at higher doses of JP-8. Further investigation is needed to verify differences in immunotoxicity results in reference to spiking methods during different seasons.

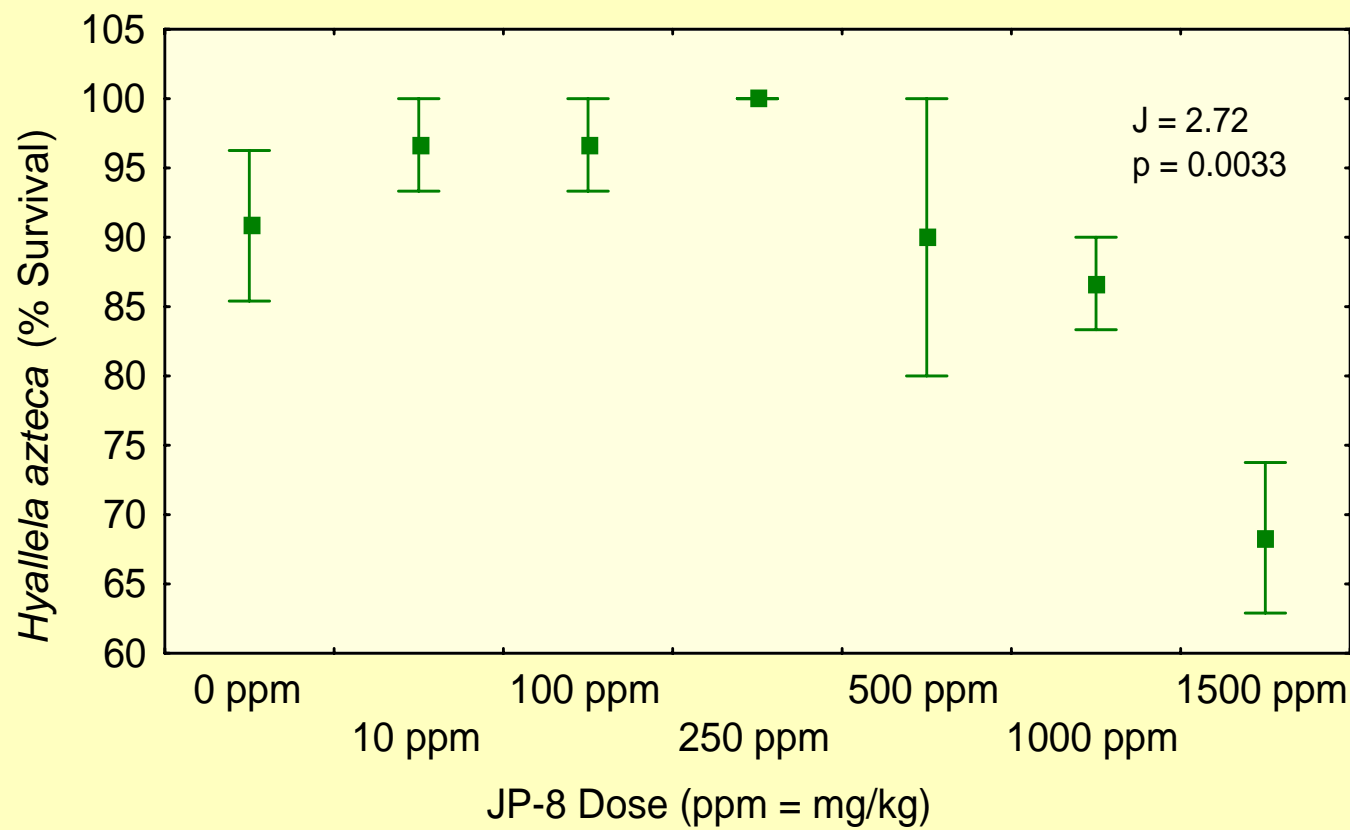
Most spiking methods have used single compounds. When spiking complex mixtures, direct spiking causes the least alteration of chemicals. Chemical analysis of TPHs may not detect lower levels of chemicals that still have biological effects on organisms. It is unclear the extent to which the Microtox<sup>®</sup> Solid-Phase Test results can be used to assess JP-8 contamination. Microtox<sup>®</sup> results suggest this assay should not be used to assess TPH contamination. Of all the responses that were measured, the *E. foetida* and *H. azteca* were the most sensitive, while *L. terrestris* and *L. sativa* were the least sensitive measures.

**Fig. 2.1.** Effect of JP-8 on the survival of *Chironomus tentans*. *C. tentans* were exposed to JP-8 in spiked sediment for 7 days, and survival was assessed. Closed circles indicate mean response for each dose. Error bars indicate one standard deviation of the mean.





**Fig. 2.2.** Effect of JP-8 on the survival of *Hyallela azteca*. *H. azteca* were exposed to JP-8 in spiked sediment for 7 days, and survival was assessed. Closed circles indicate mean response for each dose. Error bars indicate one standard deviation of the mean.



**Fig. 2.3.** Effect of JP-8 on the seed germination of *Lactuca sativa*. *L. sativa* were exposed to JP-8 in spiked soil for 5 days, and survival was assessed. Closed circles indicate mean response for each dose. Error bars indicate one standard deviation of the mean.

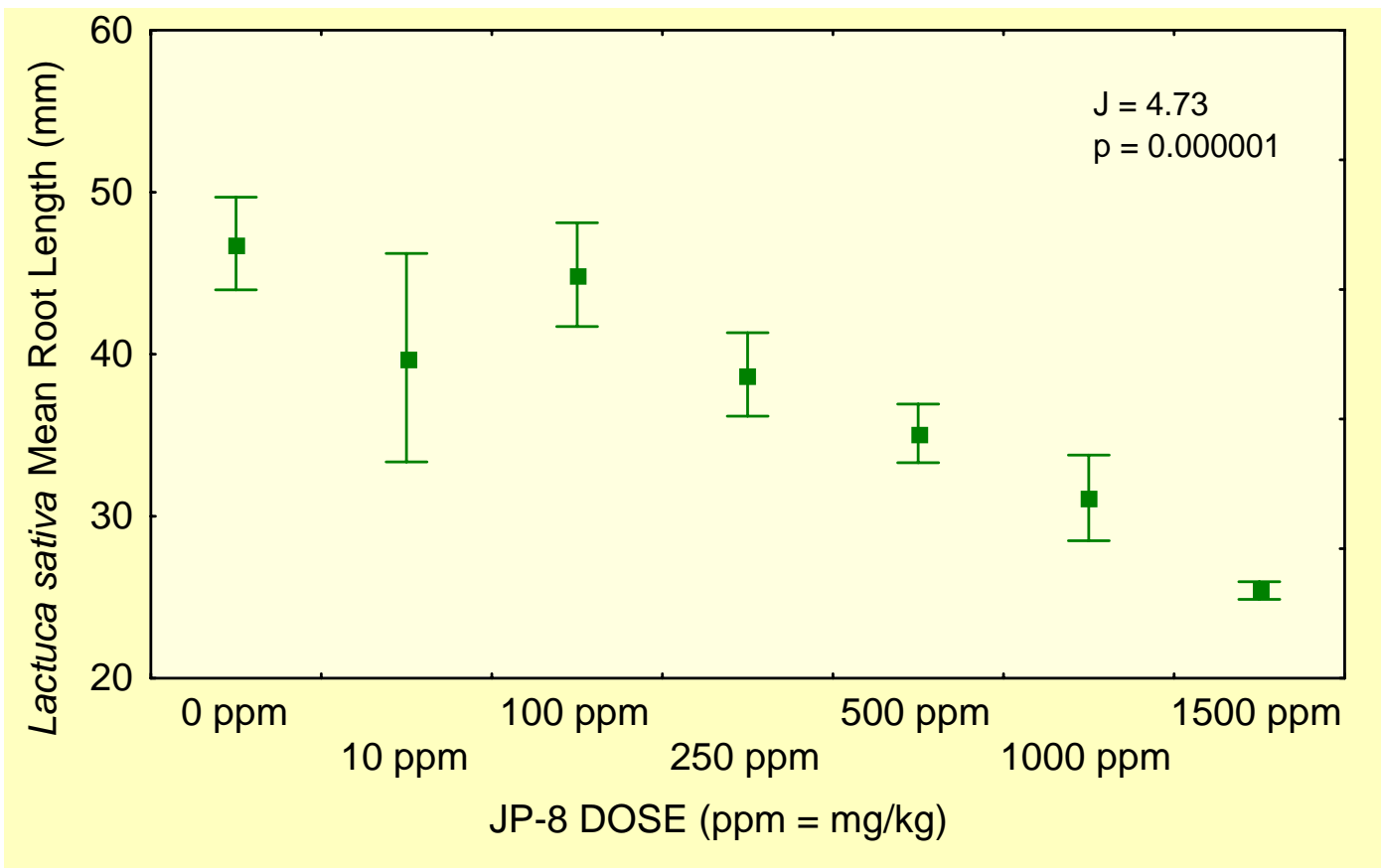


Fig. 2.4 Effect of JP-8 on mortality of *Eisenia foetida* using the No carrier / No drying spiking method. Closed squares indicate mean response for each dose. Error bars indicate one standard deviation of the mean. \* indicates  $p < 0.05$  versus control.

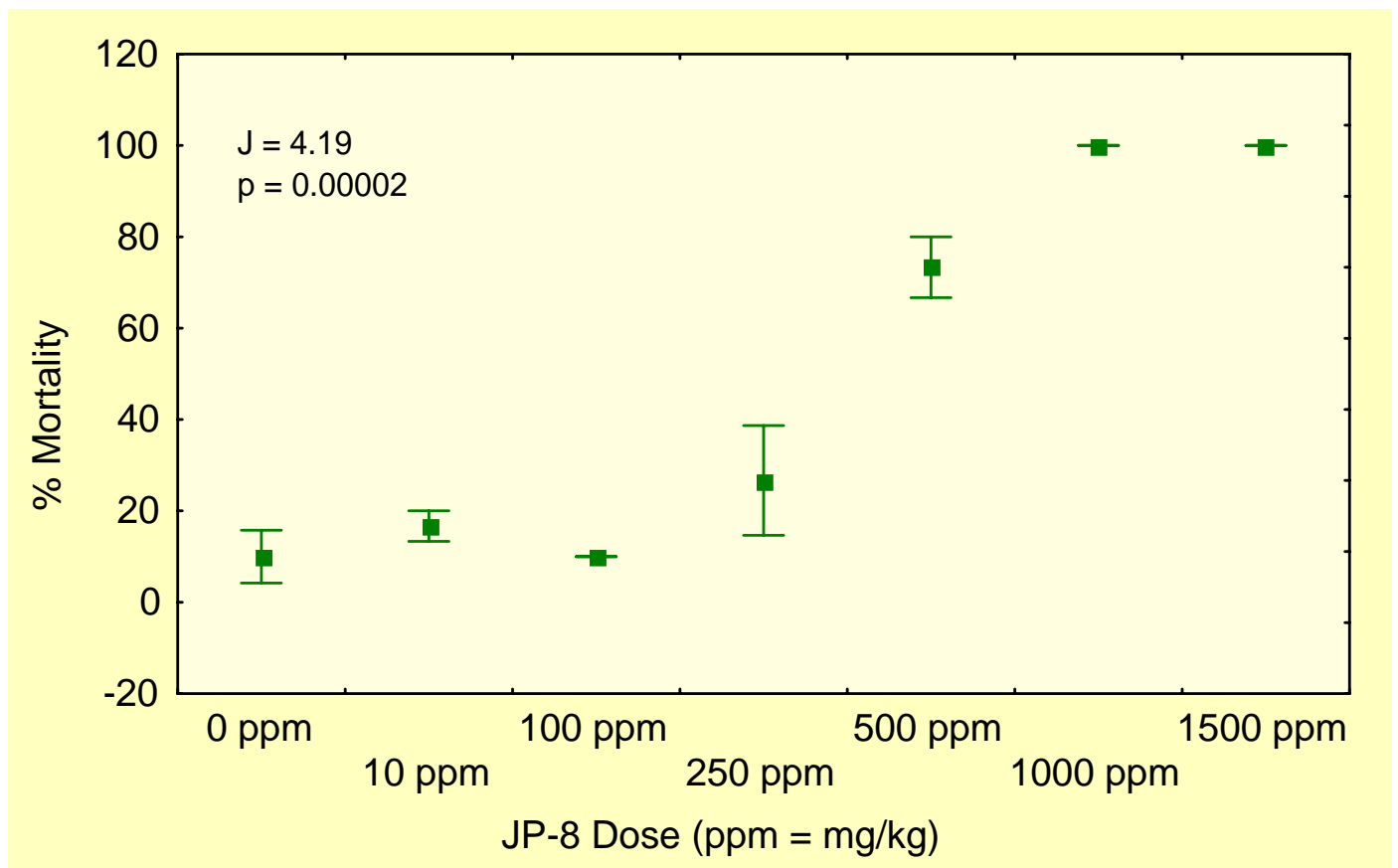
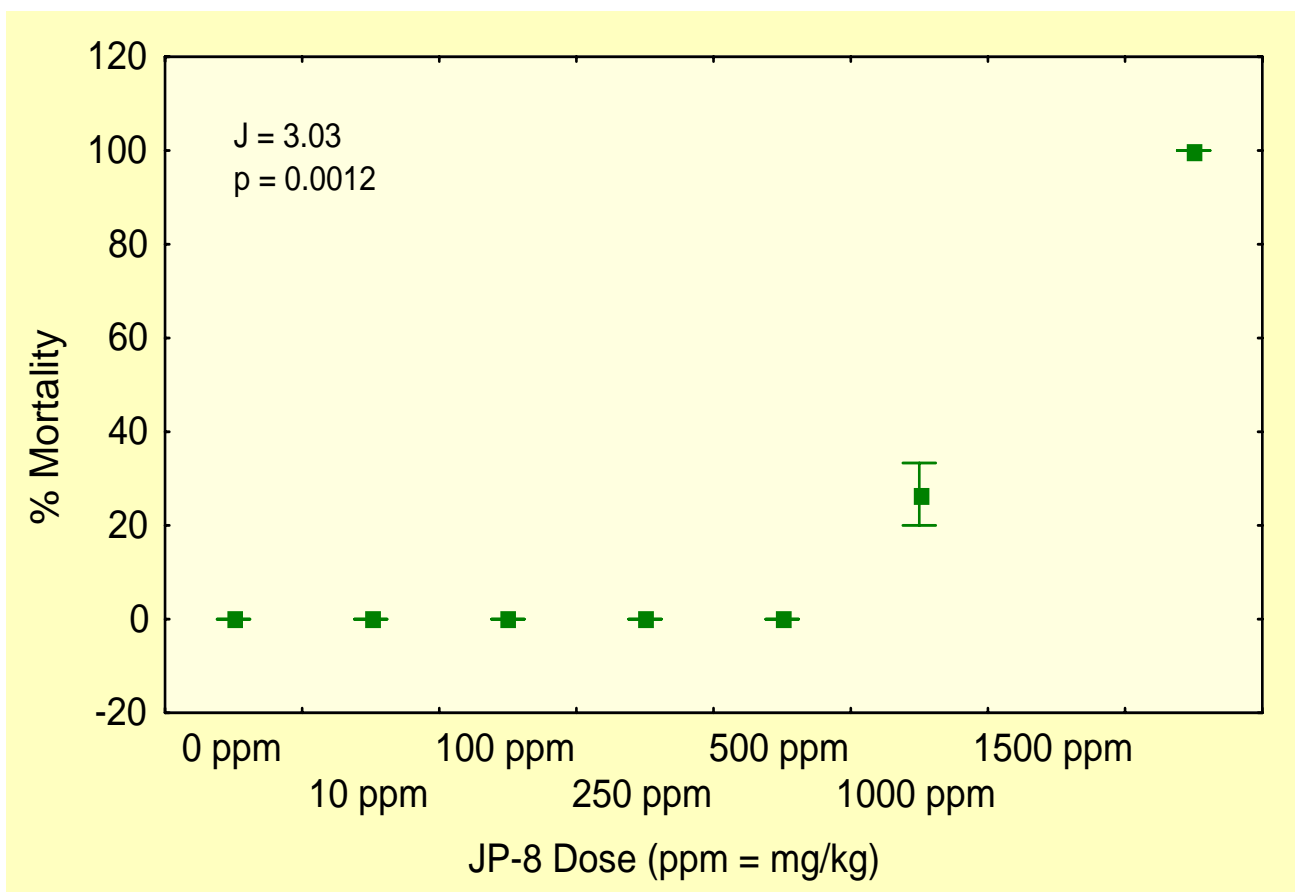


Fig. 2.5 Effect of JP-8 on mortality of *Lumbricus terrestris* using the No carrier / No drying spiking method. Closed squares indicate mean response for each dose. Error bars indicate one standard deviation of the mean. \* indicates  $p < 0.05$  versus control.





## Chapter 3.

### Effects of JP-8 Jet Fuel on the Immune Function of *Eisenia foetida*

#### Summary

This experiment evaluated the immunotoxic effects of JP-8 jet fuel on *Eisenia foetida* (redworms). A modified standard USEPA 14-day protocol was used for acute toxicity testing in 4 experiments. Soil was spiked directly with JP-8 jet fuel (nominal concentrations from 0 – 1500 ppm). Coelomic fluid was obtained using the extrusion method, and coelomocyte number and viability were assessed. Three immunoassays were used to test innate and humoral immune function. For phagocytosis, cells were incubated with fluorescent *Escheria coli* suspension for 2 hours. Uningested *E. coli* were quenched with trypan blue before fluorescence was read. For respiratory burst, cells were stimulated with phorbol myristate acetate (PMA) and incubated with a substrate (2',7' - dichlorodihydrofluorescein diacetate) that fluoresced following the respiratory burst. For secretory rosetting, coelomocytes were incubated with sheep red blood cells (SRBC) for 24 hours, and the number of rosettes (coelomocytes binding two layers of 4 or more SRBCs) was counted. JP-8 did not significantly affect the number of total viable coelomocytes or percent viability in experiments 2 and 3. However, in experiments 1 and 4, the total viable coelomocytes and the percent viability significantly decreased at and above 100 ppm. JP-8 did not affect phagocytosis (only conducted in expts. 3 & 4) in experiment 3 but was significantly decreased in experiment 4. Respiratory burst declined at and above 250 ppm in all four experiments, but was only significantly decreased in experiment 3. The number of secretory rosettes significantly decreased at and above 100

ppm. Overall, secretory rosetting was the most sensitive immunoassay. Formation of secretory rosettes results from the coelomocyte secretion of a humoral agglutinin in response to incubation with SRBCs. JP-8 greatly affected coelomocyte agglutination function. JP-8 decreased coelomocyte viability and secretory rosetting at doses that caused nominal (2.5 – 10%) mortality (100 ppm), with suppressed respiratory burst at doses (250 – 500 ppm) that caused low to high (30-95%) mortality.

## **Introduction**

Earthworms are good bioindicators of the effects of hazardous chemicals on the terrestrial environment (Rodriguez-Grau et al., 1989). Earthworms burrow through the soil and are exposed to soil contaminants dermally and through ingestion. Earthworms are an important source of food for other organisms, raising the potential for transfer of chemicals to higher trophic levels (Edwards and Bohlen, 1996). Ecotoxicological assessments mostly involve lethal toxicity testing, but many tests are being developed to assess sublethal toxicity such as behavioral, reproductive, and immunotoxic effects. In acute toxicity testing earthworms are exposed to a chemical in diluted hazardous waste soils or artificial soil, and their mortality is determined after 7 or 14 days (U.S. E.P.A., 1988). Several types of chemicals such as metals and polycyclic aromatic hydrocarbons (PAHs) have been evaluated in acute toxicity studies with earthworms. PAHs, including phenanthrene and fluoranthene, caused no earthworm mortality in contaminated forest soil from various sites near a blast furnace plant (Ma et al., 1994). Earthworms burrowing activity enhanced the disappearance of PAHs in soil. Spiking methods are

crucial with volatile compounds that can evaporate quickly. Chemical analysis can determine the actual concentrations of chemicals in soil. However, chemical analysis may not detect lower levels of chemicals that may still have biological effects on organisms.

Toxic chemicals affect the viability and functions of immune cells. Specific immune responses can be used as sensitive biomarkers for assessing the toxicity of chemicals. Coelomocytes resemble mammalian leukocytes, since they search out, phagocytize, and destroy foreign material. They also initiate inflammation, graft rejection, lysozyme production, and agglutinin secretion. Based on morphology, there are three major types of earthworm blood cells: hyaline amoebocytes (basophils and neutrophils), granular amoebocytes (granulocytes, and acidophils), and eleocytes (chloragogen cells). Basophils function in humoral-mediated immunity (HMI), which characterizes the annelids defense reactions that rely on proteolytic enzymes (Vetvicka and Sima, 1998). Acidophils function in phagocytosis (non-specific immunity - NSI), which is a first line of defense. Neutrophils, granulocytes, and chloragogen cells are transitional cells that have not yet matured into other cell types. Unlike vertebrate leukocytes, coelomocytes do not possess antibodies or T-cells. Coelomocytes produce soluble agglutinins in response to antigens, which are functionally analogous to antibodies in mammals (Goven et al., 1991).

In order to evaluate immune function in earthworms total cell counts (TCC), differential cell counts (DCC), and coelomocyte viability can be monitored easily as biomarkers for immunotoxicity testing (Cooper et al., 1996). Structural immune function can be assessed using immunoassays, which screen chemicals for suppression of humoral-mediated immunity, non-specific immunity, and cell-mediated immunity (CMI). HMI involves the elimination of extracellular pathogens by production of large numbers of proteolytic enzymes specific for antigenic determinants on foreign pathogens. NSI involves the basic resistance to disease as a first line of defense until acquired immune response develops. HMI can be assessed using hemagglutination of sheep red blood cells or secretory rosette formation with sheep or rabbit red blood cells. NSI responses can be evaluated by testing phagocytosis of fluorescent beads or yeast cells and respiratory burst. CMI involves specific recognition and selective elimination of foreign microorganisms and molecules (Kuby, 1997). CMI function can be assessed with tissue transplantation and graft rejection tests (Venables et al., 1992).

The objective of this study was to determine the lethal and sublethal toxicity of JP-8 jet fuel to earthworms. Immunotoxic effects were assessed by coelomocyte counts and three immunoassays. The total cell counts helped to determine immune suppression, and the differential cell counts determined the effects of JP-8 jet fuel on specific types of coelomocytes. A battery of immunoassays was used to detect subtle changes in coelomocyte immune function. Phagocytosis and respiratory burst assays determined the effects of JP-8 jet fuel on non-specific immunity. Secretory rosetting determined the effects on humoral-mediated immunity.

## **Materials and Methods**

### **Soil Spiking**

Artificial soil (70% silica sand, 20% kaolinite clay, and 10% peat moss) was used as the testing medium for acute toxicity testing of earthworms (USEPA, 1988). The soil was spiked directly (without a carrier) with JP-8 jet fuel. The appropriate amounts of soil were weighed out and placed in 3.785 liter (1 gallon) Nalgene containers. Four different concentrations of JP-8 were used to spike the artificial soil (0 ppm (control), 100 ppm, 250 ppm, and 500 ppm). Soil for each concentration was spiked at the same time. The containers were sealed and agitated manually for 1 minute. Next the containers were mechanically shaken at 300 rpm for 24 hours. Control soils were agitated manually and mechanically shaken, but did not receive any concentrations of JP-8. For all soil tests, soil moisture and pH were measured at the beginning and end of the experiment.

### **Culturing and Collection of Organisms**

Mature clitellate redworms (*Eisenia foetida*) were purchased from Carolina Biological Supply and acclimated in the dark to  $20 \pm 2$  °C in an environmental chamber for at least 2 weeks before testing.

## Exposure

Acute toxicity tests were done in triplicate with 10 *E. foetida* (redworms) (200-300 mg) and 200 g dry weight of soil per 1 pint mason jar (USEPA, 1988). Earthworms were randomly selected and placed into each treatment jar. Temperature was maintained at  $20 \pm 2^{\circ}\text{C}$  for *E. foetida* in an environmental chamber. Continuous light was used to encourage burrowing of worms. Mortality was assessed after 7 days of exposure.

## Coelomocyte Counts

Coelomocytes were obtained from survivors of acute toxicity tests. *E. foetida* were rinsed with  $4^{\circ}\text{C}$  saline to remove dirt. *E. foetida* cells were obtained using the extrusion method. Two to four *E. foetida* were placed into a 1.5 ml microcentrifuge tube, and 0.5 ml of extrusion fluid (8.357g NaCl, 0.4217g  $\text{CaCl}_2$ , 0.2711g KCl, 0.0544g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0804g  $\text{KH}_2\text{PO}_4$ , 0.3528g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.3578g  $\text{NaHCO}_3$  per liter of distilled water) was added to the tube. After 4 minutes of incubation, the worms were removed from the tube, and the extruded cells (contained in 0.5 ml of extrusion fluid) were diluted with 0.5 ml of *Lumbricus* Balanced Salt Solution w/o  $\text{CaCl}_2$  (LBSS w/o  $\text{CaCl}_2$ ). Cells from *E. foetida* were recovered by centrifugation at  $150 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  and resuspended in 0.5 ml of LBSS w/ $\text{CaCl}_2$  at  $4^{\circ}\text{C}$ . Cell numbers and viability were determined by counting in a hemacytometer at 400x using trypan blue viability dye. The following endpoints were assessed: number of total coelomocytes, number of live coelomocytes, and percent viability of coelomocytes. A coelomocyte smear was stained with Wright Stain (Sigma Chemical Co., St. Louis, Missouri), and differential coelomocyte counts were conducted at 1000x using oil immersion.

## Immune Function Tests

The ability to phagocytize fluorescent particles can indicate the sublethal immunotoxic effects of JP-8. For phagocytosis, cells were incubated with fluorescent *E. coli* bioparticle (Molecular Probes Inc., Eugene, Oregon) suspension. After incubation, non-phagocytosing cells were quenched with trypan blue solution. Fluorescence was measured at 480 nm excitation and 520 nm emission with a microplate reader (Fluostar) to determine the uptake of fluorescent particles.

Once particles are phagocytized they are destroyed by reactive oxygen species (ROS). The effects of JP-8 on coelomocyte ability to produce oxidative chemicals were assessed using the fluorescent probe 2',7' – difchlorofluorescein-diacetate (H<sub>2</sub>DCFDA) which has not previously been used with earthworm coelomocytes. For respiratory burst, coelomocytes (0.1 ml) in LBSS w/ CaCl<sub>2</sub>, 0.01 ml PMA (2ng/ml) (Sigma Chemical Co., St. Louis, Missouri) and 0.005 ml of 5 mM H<sub>2</sub>DCFDA (Molecular Probes Inc., Eugene Oregon) per well were added to the 96-well microplate for experimental wells (Rosenkranz et al., 1992). Immediately the plate was read at 538 emission and 485 excitation for a one-hour kinetic reading (spectrofluorometer Fluostar).

The ability to recognize non-self and form agglutinins is an important immune function. Secretory rosetting was used to determine the effects of JP-8 on coelomocyte agglutinin function. To begin secretory rosetting, 0.1 ml aliquots of RRBCs suspension were added to equal volumes of coelomocytes in 1.5 ml microcentrifuge tubes and



centrifuged at 100 x g for 5 minutes. After incubation for 24 hours at 10<sup>0</sup> C, a 0.1 ml aliquot of supernatant was taken from each tube and mixed with 0.1ml of crystal violet in LBSS solution. The crystal violet stained the rosettes that formed. The number of (rosettes) coelomocytes binding two or more layers of at least four erythrocytes were counted (Rodriguez-Grau et al., 1989).

## **Statistical Analysis**

The immunotoxic effects of JP-8 were analyzed using one-way analysis of variance (ANOVA) (Statistica, City, TX). The non-parametric Jonckheere test for ordered alternatives was used to evaluate monotonic trends between dose groups and dependent variables (Hollander and Wolfe, 1973). The Dunn's test was used to compare each dose to controls when the Jonckheere test indicated a significant dose response. The Dunn's test indicates one-way differences between control groups and other groups. Both the Jonckheere and Dunn's test allow for unequal sample sizes and are corrected for ties.

## **Results**

### ***Mortality***

No mortality occurred in any of the control treatments for all 4 experiments. There was no mortality in the 100 and 250 ppm nominal doses for experiments 3 and 4. There was low to moderate (5-27.5%) mortality for experiments 1 and 2 at the 250 ppm nominal dose. There was low (10%) mortality at the 500 ppm nominal dose for experiment 2 and high (95 – 100%) mortality for all other experiments. Mortality was also 100% for the 1000 ppm nominal dose in experiment 4 only (Table 3.1). The body

masses for *Eisenia foetida* used in all 4 experiments were  $\geq 300$  mg in experiment 1;  $\geq 400$  mg in experiment 2; and  $\geq 350$  mg in experiments 3 and 4 (Table 3.1).

### **Immunotoxicity**

In experiments (1 and 4) with low to moderate (0-27.5%) mortality at the 100 and 250 ppm nominal doses, there were significant decreases in total viable cells and percent viability (Table 3.1). There were no significant decreases in total viable cells and percent viability in experiments (2 and 3) that had low (0-5%) mortality at the 100 and 250 ppm nominal doses (Table 3.1). Experiment 2 also had low (10%) mortality at the 500 ppm nominal dose. JP-8 did not affect phagocytosis in experiment 3. There was a significant decreasing trend in phagocytosis for experiment 4 (Table 3.2). Respiratory burst was not affected in experiments 1, 2, and 4 (Table 3.2). However, there was a decline in respiratory burst at and above 250 ppm for experiment 3 (Table 3.2). Differential cell counts showed that the coelomocytes responsible for phagocytosis (acidophil numbers and percent) increased, but the phagocytosis assay showed that even though more phagocytic cells were present, they were not functional (Fig. 3.2). The percentage of transitional cells (neutrophils, granulocytes, and chloragosomes) also increased, while the percentage of basophils decreased.

Overall, secretory rosetting was the most sensitive immunoassay. In all experiments where secretory rosetting was measured (Expts. 1, 2, and 3) JP-8 greatly affected agglutination function (Table 3.2). There was a very significant decreasing trend in the formation of rosettes beginning at doses that caused nominal (0-2.5%) mortality (100 ppm) and continually declining at doses (250-500 ppm) that caused moderate to high mortality (27.5-100%) (Table 3.2).

## Discussion

Acute toxicity exposure to JP-8 resulted in mortality and sublethal toxicity in *E. foetida*. Worm size can influence the toxicity of JP-8. The USEPA standard acute toxicity protocol requires worms of 300 – 500 mg weights to be used in testing (USEPA, 1988). Most of the worms used in these experiments were on the lower end of this range (300-350 mg). Differences in immunotoxicity results could be related to worm size. The overall higher mortality in experiment 1 was most likely due to the use of smaller ( $\geq 300$  mg) worms. For experiments 1 and 4, there were significant decreasing trends in immunoassays. In experiments 2 and 3 there was low mortality at 100 and 250 ppm and there were no significant decreasing trends in immunoassays. Overall, differences in mortality from 250-500 ppm between experiments, as well as insignificant effects in cell viability, total cell viability, and immunoassays for experiments 2 and 3 could be due to differences in worm size or overall health of the worms. Experiment 2 used the largest worms ( $\geq 400$  mg) and had the lowest mortality of all the experiments, as well as no significant immunotoxic effects. Larger worms appear to be less susceptible than smaller worms to nominal doses (100-1000 ppm) of JP-8.

Spiking methods can also influence mortality results as well. Direct spiking methods were used to incorporate JP-8 into the soil. Previous experiments have shown that direct spiking results in the highest retention of actual TPH concentrations in soil when spiking volatile chemicals (unpublished data). Based on chemical analysis, actual concentrations of JP-8 in soil can be as much as 10 times lower than the nominal dose

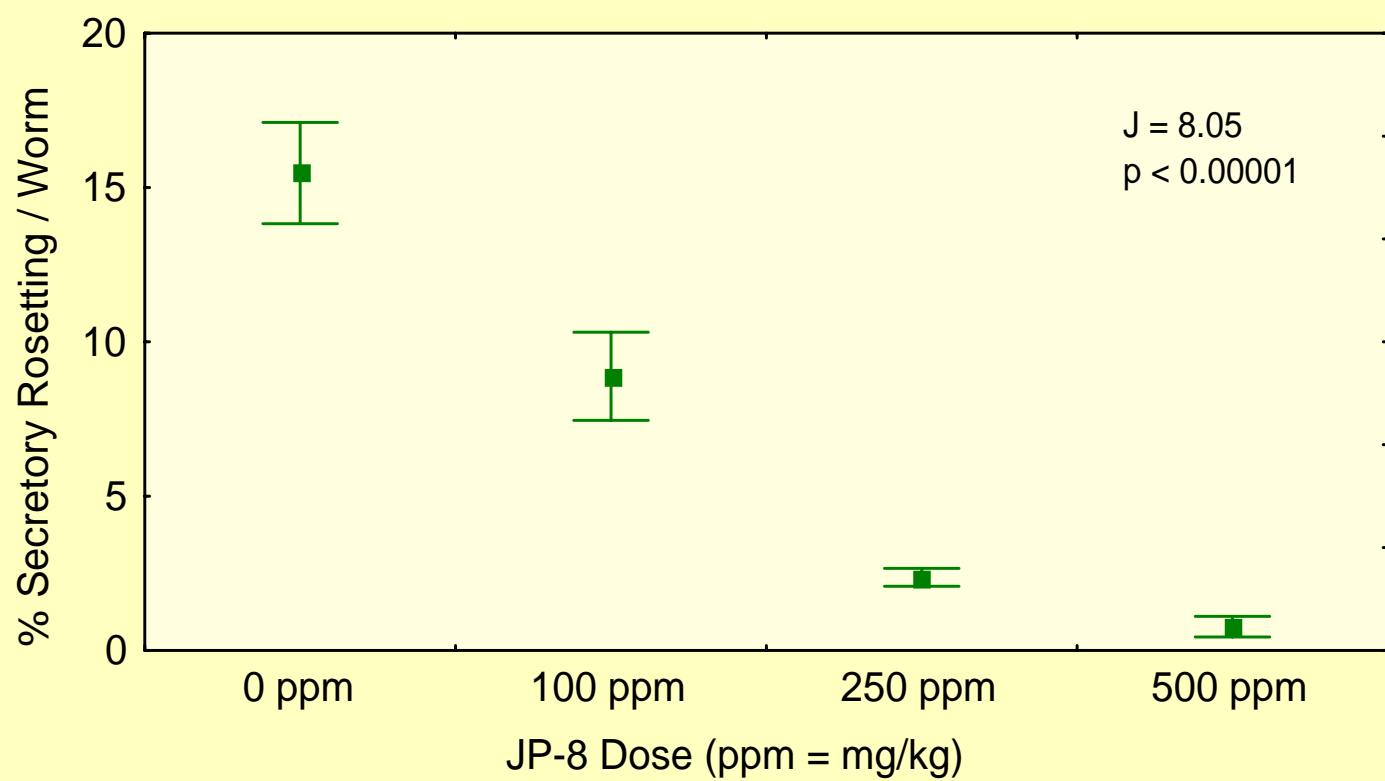
(e.g., nominal 1000 ppm = actual 100 ppm) (unpublished data). Even though the actual concentrations are low (from a chemical standpoint), significant biological effects (such as mortality and immunotoxicity) can still be found.

Mortality was seen at nominal concentrations of 250-1000 ppm. Decreases in total cell viability and % viability at concentrations of 250-1000 ppm explain the significant ( $p \leq 0.05$ ) decreases in secretory rosetting (expts. 1-3), respiratory burst (expt. 3), and phagocytosis (expt. 4). Increases in phagocytic cells (acidophils) were detected in differential cell counts. Results of the phagocytosis assay show that these cells (although more proliferative) were rendered non-functional by JP-8.

Immunoassays can detect more sensitive changes in coelomocyte function than cell counts. Phagocytosis is a first line of defense in non-specific immunity, which makes it a good assay for endpoints in immunological studies. In earthworms, it is the basic mechanism for the clearance of intruders (bacteria, debris) in the body cavity fluid (Jarosz and Glinski, 1997). Amoebocytes from many earthworm families participate in the various steps of phagocytosis which involve recognition, chemotaxis, attachment, ingestion, and intercellular killing of biotic objects by lysosomal enzymes (Jarosz and Glinski, 1997). Other biomarkers such as neutral red retention (NRR) have been used to assess the immunotoxic effects of copper on earthworms (Scott-Fordsmand, Weeks, Hopkin, 2000). The NRR-times for *E. fetida* were depressed at lower concentrations in laboratory spiked soils than in field contaminated soils (Scott-Fordsmand, Weeks, Hopkin, 2000).

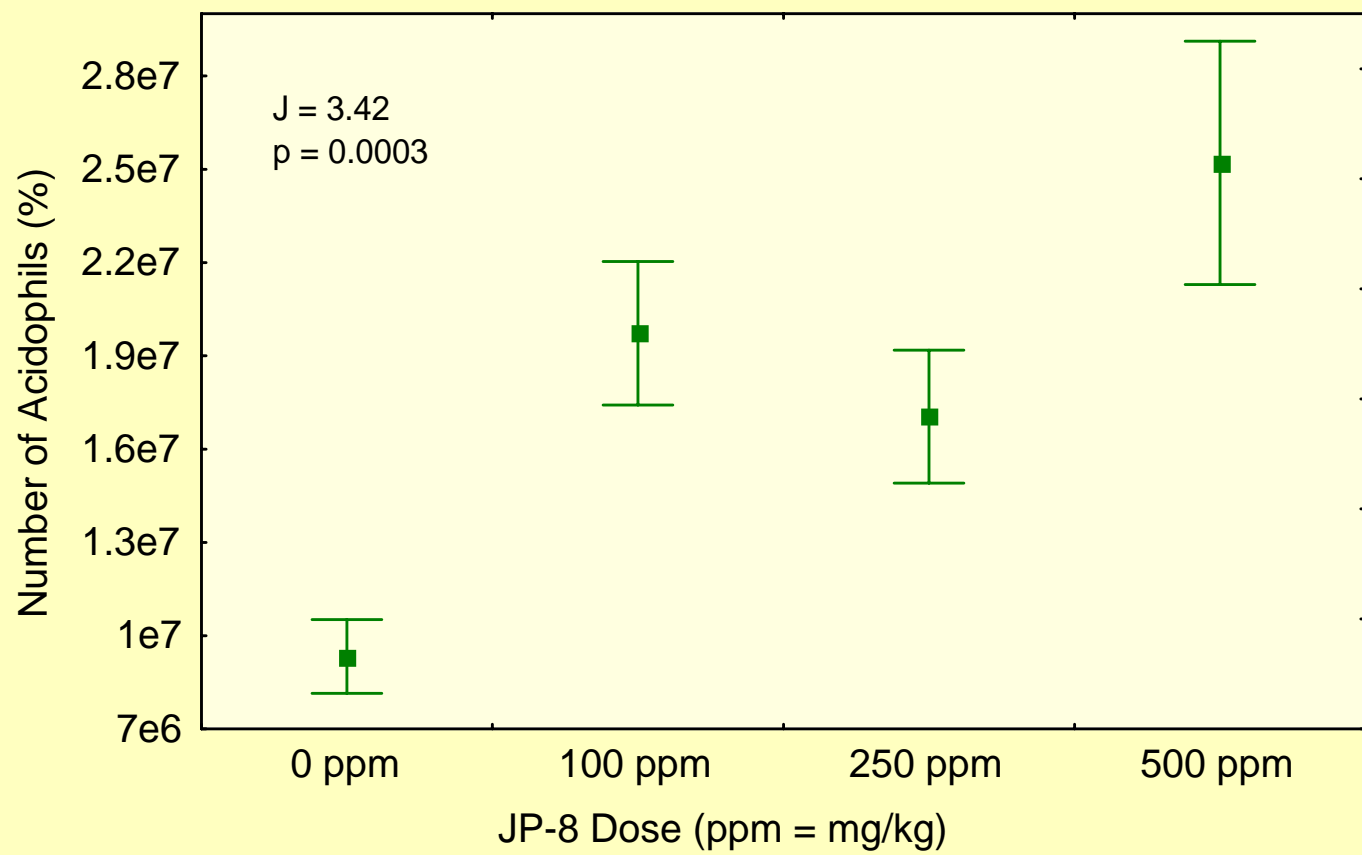
Along with phagocytosis, the ability of earthworm coelomocytes to produce reactive oxygen species and destroy engulfed particles as an immune defense is important (Valembois and Lassegues, 1995). In vertebrates, phagocytosis stimulates reactive oxygen species (ROS) to produce and exert a cytotoxic action on pathogens. ROS activity on lipids leads to an accumulation of lipofuscin brown bodies (Valembois and Lassegues, 1995). Secretory rosetting helps to evaluate whether or not cells have the ability to recognize non-self. The significance of rosette-forming cells in invertebrates is not as well known as in vertebrates. Rosette-forming cells in vertebrates possess receptors that recognize antigen and binds them to their surfaces (Cooper, 1976). In invertebrates, coelomocytes actively secrete a synthesized humoral agglutinin factor in response to antigens, which forms secretory rosettes (Eyambe et al., 1991). Overall, secretory rosetting was the most sensitive immunoassay of those used to evaluate the immunotoxic effects of JP-8 on earthworms.

**Fig. 3.1.** Effect of JP-8 on the ability to form secretory rosettes in *E. foetida*. Earthworms were exposed to JP-8 in spiked artificial soil for 7 days, and secretory rosetting was assessed on day 7 of exposure. Closed circles indicate mean response for each dose. Error bars indicate one standard deviation of the mean.



**Fig. 3.2.** Effect of JP-8 on the phagocytic cell subpopulation (acidophils) of coelomocytes in *E. foetida*. Earthworms were exposed to JP-8 in spiked artificial soil for 7 days, and differential cell counts were performed on prepared slides. Closed circles indicate mean response for each dose. Error bars indicate one standard deviation of the mean.





## Chapter 4.

### Summary and Conclusions

The experiments reported in Chapters 2 and 3 demonstrated the effects of different spiking methods on the acute toxicity and immunotoxicity of JP-8 to various aquatic and terrestrial organisms. The results of chapter 2 concurred with spiking method findings that directly spiked soil requires the least handling to reduce the loss of chemicals (Northcott and Jones, 2000). There is some question about what constitutes the best spiking methods. Several factors can affect the incorporation of a chemical into soil such as the type of soil and homogeneity, the type of chemical, carriers, and handling (Northcott and Jones, 2000). All of these factors can greatly affect the results of acute toxicity and immunotoxicity of chemicals. More studies using different spiking methods with various chemicals are needed to standardize procedures.

The acute toxicity of chemicals can be evaluated best in various organisms. There is a great need for a test battery approach in order to assess multiple species, and multiple endpoints (Pauwels, 1999). The results of chapter 2 demonstrated this need with the responses of various organisms to soils spiked with JP-8. *C. tentans* had high mortality in all treatments which does not make them the best indicators of toxicity. However, *H. azteca* appear to be a better choice in aquatic organisms for testing the toxicity of JP-8. For terrestrial organisms *E. foetida* and *L. sativa* are the better indicators of JP-8 toxicity. Overall, *L. terrestris* were less sensitive to the effects of JP-8.

Evaluating the sublethal effects of chemicals can be a more sensitive endpoint than acute toxicity. Acute toxicity effects can possibly predict the immunotoxic effects of chemicals. In previous experiments, high mortality resulted in high immunotoxicity (Handy and Grasman, unpublished data). Experiment 1 in Chapter 3 concurs with this hypothesis. There are also other factors such as worm size, overall health, and test methods that can affect the acute toxicity and immunotoxicity of JP-8. Some immunoassays are more sensitive than others as well. This research showed that secretory rosetting is a very sensitive indicator of immune effects of chemicals, which concurs with previous findings (Goven et al., 1993, Venables et al., 1992, and Weeks et al., 1992).

More studies are needed to increase knowledge about the effects of spiking methods on acute toxicity, species sensitivities, and immunotoxicity of chemicals. Different types of chemical analysis could be employed to determine actual concentrations of chemicals. The direct spiking method could be used with various chemicals and various soils to determine differences in retention of chemicals. The use of multiple species with similar sensitivities could better evaluate the effects of chemicals. Multiple habitat levels and routes of exposure should be considered as well (Pauwels, 1999). Several other areas could be pursued such as different immunoassays. For instance, flow cytometry could further elucidate the types of coelomocytes functioning in various immune responses. The use of earthworms in immunotoxicity testing and extrapolation to other organisms could be investigated as well. This research project briefly touched various questions about spiking methods, acute toxicity, and immunotoxicity. Clearly, there is a broad

spectrum of research that is needed to evaluate the fate of chemicals in soil and sediment habitats.

Finally, earthworms are good models for acute toxicity and immunotoxicity testing. They are easy to work with, reproducible, cost-efficient, and noncontroversial research organisms. Some mechanisms of earthworm immune function are not known which could make extrapolation of data to mammals and humans difficult (Goven et al., 1993). Earthworms and lettuce seeds (terrestrial) are more reliable than aquatic organisms for testing soil spiking methods. Spiking methods affect the amount of chemical incorporated into soil and need to be considered when spiking with volatile compounds. The method used for spiking can greatly affect the biological results in laboratory experiments. Furthermore, future studies are needed to increase the knowledge base of what data can be obtained from using different spiking methods and immunotoxicity in earthworms.

## **Appendix**

### **Experimental Design for Acute Toxicity Testing**

Soil testing exposed worms to various concentrations of jet fuel, and provided controls and survivors for immunotoxicity testing. Soil testing was done to optimize spiking methods and some immunoassays such as total and differential cell counts were done as part of these tests. After immunoassays were optimized, more soil testing was done with total and differential cell counts as well as phagocytosis, oxidative burst, and secretory rosetting immunoassays. Definitive soil tests used the best spiking method and the best worm species as determined in preliminary testing.

### **Soil Testing**

To determine the effects of spiking methods on soil toxicity, three spiking methods were used in preliminary testing (hypothesis 1). The different spiking methods evaluated the loss of volatile components of JP-8 jet fuel during the spiking process. One method used acetone carrier and drying in the fume hood, another method directly spiked the soil and was also dried, and the other method directly spiked the soil without drying. The soil for the two hood-dried spiking methods was rehydrated, while the other method did not require re-hydration. Three experiments using five doses of jet fuel in triplicate and both *L. terrestris* (5 worms per dose) and *E. foetida* worms (10 worms per dose) optimized spiking methods. Chemical analyses were done on pre and post acute toxicity test soil samples to determine the amounts of JP-8 lost due to volatilization.

Using both species of worms and determining mortality helped to determine species sensitivities to jet fuel and address hypothesis 2. After determining the best spiking method, 3 definitive tests will be done in triplicate with either 10 *E. foetida* worms or 5 *L. terrestris* worms and 200 g dry weight of soil per 1 pint mason jar. For all soil tests, soil moisture and pH will be measured at the beginning and end of the test. Mortality will be checked after 7 and 14 days of exposure.

At the end of each acute toxicity test, total cell counts and differential cell counts will be conducted on controls and survivors. Following definitive tests, cell concentrations will be adjusted and phagocytosis, oxidative burst, and secretory rosetting immunoassays on cells obtained from the controls and survivors exposed to low doses of jet fuel will be conducted to determine immune suppression (hypothesis 3).

### **Immunotoxicity Testing**

These various immunoassays will address hypothesis 3.

Initially, total cell counts and differential cell counts will be conducted on controls and survivors (exposed to low and medium doses of jet fuel) of the acute soil toxicity and contact filter paper tests. The total cell counts will help to determine immune suppression, and calculate cell concentrations for other immunoassays, and the differential cell counts will determine the effects of JP-8 jet fuel on specific types of coelomocytes.

A battery of assays using three immunoassays: phagocytosis, oxidative burst, and secretory rosetting will determine the immune suppression of worms exposed to JP-8 jet fuel. Phagocytosis, and oxidative burst assays will determine the effects of JP-8 jet fuel on non-specific immunity. Phagocytosis is one of the first lines of defense for eliminating bacteria and other pathogens. All coelomocytes except chloragogen cells are phagocytic. This test will determine the phagocytic activity of coelomocytes from control worms and worms exposed to either jet fuel. Cell concentrations will be adjusted to  $1 \times 10^6$  cells/ml for phagocytosis assays. Phagocytosis assays will be done in duplicate on the cells of either one *L. terrestris* worm, or the pooled cells of four *E. foetida* worms. Once pathogens are phagocytized an oxidation reduction reaction must occur so that the pathogens can be destroyed. Oxidative burst assays will determine the ability of coelomocytes to oxidatively kill phagocytized bacteria. Cell concentrations will be adjusted to  $1 \times 10^5$  cells/ml for NBT assays. The oxidative burst assays will be done in

triplicate on the cells of either one *L. terrestris* worm, or the pooled cells of four *E. foetida* worms.

Secretory rosetting will determine the effects of jet fuel on humoral-mediated immunity. Secretory rosetting assays will be used to determine the ability of coelomocytes to produce agglutination factors in response to a foreign challenge. Cell concentrations will be adjusted to  $1 \times 10^6$  cells/ml for secretory rosetting assays. Secretory rosetting assays will be done in duplicate on the cells of either one *L. terrestris* worm, or the pooled cells of four *E. foetida* worms.

## **VII. Specific Methods**

The following specific procedures will be used in these various tests and assays to address hypotheses 1, 2, and 3.

### **Exposure Protocols**

#### **14-Day Acute Toxicity Tests**

**Purpose-** To expose earthworms to various concentrations (ppm = mg/kg) of jet fuel using two different spiking methods and assess mortality. The survivors in these tests will be used for immunotoxic tests also.

#### **Procedure-**

Weigh out the appropriate amount of soil and place in 1 gal. Nalgene containers.

Spike the soil with the appropriate amount of JP-8 jet fuel (non-acetone), seal the containers and agitate manually (1 minute).

When using acetone as a carrier, dissolve the appropriate amount of JP-8 in a 10% volume soil ratio of acetone. Pour the JP-8 w/acetone carrier onto the soil in the

container, seal, and agitate manually. Place the containers on the shaker, and secure them. Set the shaker for 300 rpm and shake the soil for 24 hours. Spread the soil out onto foil and let it dry for 24 hours in the fume hood. Remove the soil from the fume hoods, and break into small clumps with a metal spoon. Weigh out the appropriate amount of soil, and rehydrate it with double distilled water. Place the soil into the 1-pint jars. Measure the pH and moisture of each treatment. Randomly select and place 10 *E. foetida* worms ( $\geq 300\text{mg}$ ) or 5 *L. terrestris* worms ( $\geq 3\text{g}$ ) into each treatment jar.

## **Immunological Assays**

### **Steps for Extrusion of Coelomocytes**

Earthworms will be rinsed with 4°C saline to cleanse the dirt off of them. The lower one-fourth posterior end of the worm will be massaged to remove fecal contents. Next, 4 earthworms will be placed into a 1.5-ml microcentrifuge tube, and 0.75 ml of extrusion fluid added to the tube. The worms will be incubated in extrusion fluid for 4 minutes at room temperature. The worms will be removed from the tube and the extruded cells (contained in 3ml of extrusion fluid) will be diluted with 1 ml of Lumbricus Balanced Salt Solution w/o  $\text{CaCl}_2$  (LBSS w/o  $\text{CaCl}_2$ ). Cells will be recovered by centrifugation at 150 x g for 15 minutes at 4 C.

Lastly, the cells will be spun two more times and then the cell pellet will be resuspended at 4 C, in 0.5 ml of LBSS w/ $\text{CaCl}_2$ .



### **Steps for Puncture Method**

Earthworms will be rinsed with 4°C saline solution to cleanse the dirt off of them.

The earthworms will be placed in a petri dish and put on an ice bath for 20 minutes.

A sharpened pasteur pipette will be inserted laterally into the area posterior (approximately 5-10 segments) to the clitellum. Coelomic fluid will flow into the pipet by intracoelomic pressure. Cells will be diluted with 0.25 ml of Lumbricus Balanced Salt Solution w/CaCl<sub>2</sub> (LBSS).

**Note: After obtaining cells with either the extrusion or puncture method Total Cell Counts and Differential Cell Counts can be done.**

### **Total Cell Counts (TCC)**

25 ul of the cells will be transferred into another test tube containing 475 ul of LBSS w/CaCl<sub>2</sub>, and 500 ul of Trypan Blue viability dye. The cells will be allowed to absorb the dye for 10 minutes at room temperature. An aliquot of 25 ul of cells will be placed into the hemacytometer, and the cells will be counted.

### **Differential Cell Counts (DCC)**

A drop of the diluted cells will be placed on a slide (using a disposable pipet) in order to make a smear. Next, the slide will be stained with Wright Stain. After slide has dried, the different cell types will be counted.

**Phagocytosis (NSI):** Using FluoReporter Assay Kit with the Fluorometer (CL).

**Purpose-** Phagocytosis is one of the first lines of defense for eliminating bacteria and other pathogens. This test will determine the phagocytic activity of coelomocytes from control worms and worms exposed to jet fuel.

**Procedure-**

Coelomocytes will be obtained from survivors of general methods tests using the puncture method. Cell concentrations will be determined with a TCC using the hemacytometer, and adjusted to  $1 \times 10^6$  cells/ml. Cells will be incubated with fluorescent *E. coli* Bioparticle suspension prior to the experiment. After incubation, non-phagocytosing cells will be quenched with 100 ul of Trypan Blue solution.

Next, the experimental and control wells of the microplate will be read in the fluorescence plate reader using 480 nm excitation, 520 nm emission, and the appropriate sensitivity settings, to determine the uptake of fluorescent particles. Lastly, the net phagocytosis and the response to chemical exposure will be calculated.

**Secretory Rosetting (HMI):** Using SRBC or RRBC with the spectrophotometer method (CL).

**Purpose-** To determine the ability of coelomocytes to produce agglutination factors in response to a foreign challenge.

**Procedure-**

Coelomocytes will be obtained from survivors of general methods tests using the extrusion method. Cell concentrations will be determined with a TCC using the hemacytometer, and adjusted to a cell concentration of  $1 \times 10^5$  cells/ml.

To begin, 0.1-ml aliquots of RRBCs suspension will be added to equal volumes of coelomocytes in 1.5-ml microcentrifuge tubes, and centrifuged at 100g for 5 minutes.

After incubation for 24 hours at 10°C, a 0.1-ml aliquot of supernatant will be taken from each tube and mixed with 0.1ml of crystal violet in LBSS solution. The crystal violet

will stain the rosettes that have formed. Next, the number of (rosettes) coelomocytes binding two or more layers of at least four erythrocytes will be counted.

**Oxidative Burst (NSI):** Using the fluorescent probe H2DCFDA and the spectrofluorometer.

**Purpose-** To determine the ability of coelomocytes to oxidatively kill phagocytized bacteria.

**Procedure-**

Coelomocytes will be obtained from survivors of general methods tests using the extrusion method. Cell concentrations will be determined with a TCC using the hemacytometer, and adjusted to a cell concentration of  $1 \times 10^6$  cells/ml. First, 100  $\mu$ l of cells in LBSS w/  $\text{CaCl}_2$  along with an additional 0.015 ml of LBSS w/  $\text{CaCl}_2$  will be added to the appropriate wells for negative-control wells (cells only). Next, 0.1 ml of cells in LBSS w/  $\text{CaCl}_2$ , 0.01 ml PMA (2ng/ml) and 0.005 ml of 5 mM H2DCFDA per well will be added to the 96-well microplate for experimental wells. Immediately the 96-well microplate will be put into the spectrofluorometer (Fluostar) and the plate was read at 538 emission and 485 excitation for a one-hour kinetic reading.

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